PRONIOSOMAL TRANSDERMAL DRUG DELIVERY SYSTEM OF ESTRADIOL, ETHINYLESTRADIOL AND LEVONORGESTREL FOR CONTRACEPTION & HORMONE REPLACEMENT THERAPY

A Thesis submitted in Partial fulfillment of the requirement
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By

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॥ श्री गणेशाय नमः ॥

Dedicated to

My

Loving Parents for being the wind beneath my wings

Declaration by the Candidate

I declare that the thesis entitled **"Proniosomal Transdermal Drug Delivery System of Estradiol, Ethinylestradiol and Levonorgestrel for Contraception and Hormone Replacement Therapy"** is my own work conducted under the supervision of **Prof. Ramesh Chandra** (Supervisor), The Vice-Chancellor, Bundelkhand University, Jhansi and **Prof. G.P. Agrawal** (Co-supervisor), Department of Pharmaceutical Sciences, Dr. Hari Singh Gour University, Sagar, at Institute of Pharmacy, Bundelkhand University, Jhansi. The same is submitted to Bundelkhand University, Jhansi for the partial fulfillment of the requirement for the award of Ph.D. degree in Pharmacy.

Rameshchandra
Signature of the Supervisor

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Signature of the Co-Supervisor

Signature of the Candidate

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Ramesh Chandra

Signature of the Supervisor

Signature of the Candidate

Certificate of the Supervisor

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To the best of my knowledge and belief the thesis:

(i) embodies the work of candidate himself;

(ii) has duly been completed;

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(iv) is upto the standard both in respect of content and language for being referred to the examiner

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Certificate of the Co-Supervisor

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Date:

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INTRODUCTION

The concept of transdermal delivery (TD) for steroid application has now-a-days been largely accepted for hormone replacement therapy in the menopause. It is only recently that the same concept has been envisaged for contraception. Both estrogens and progestogens can penetrate the skin; provided they are delivered in an appropriate solvent. About 10% of the total dose applied topically is actually absorbed. The transdermal delivery systems presently available are either reservoir type or matrix dispersion type where the drug is dispersed into a polymer matrix. Estradiol is the most appropriate steroid for TD and can be combined with progestins to ensure a contraceptive effect. Only potent progestins should be used to achieve effective plasma levels with low doses in order to maintain an acceptable small surface of TDS.

(A) HORMONE REPLACEMENT THERAPY

At menopause, the cessation of ovaries secretion of estradiol initiates the train of events unique to the human female. For many women, the symptoms of menopause (such as vasomotor instability, vaginal discomfort, and mood variation) are not severe or prolonged and usually do not necessitate estrogen treatment. The acceleration of bone mineral loss by estrogen deficiency, however, contributes substantially to osteoporosis, which is present in virtually all women who are 50 years old or more. The frequency of spinal and hip fractures progressively increases as the years after menopause in advance. Thus, in most women, estrogen treatment becomes an important consideration for both prevention and control of osteoporosis.

Many important questions about estrogen treatment can be posed. What type of estrogen treatment (oral, injectable, transdermal) is best? What dosage should be used, at what age should treatment begin, and how long should treatment be continued? Which indication should prompt estrogen therapy? What risk to be benefit ratio can be cited for the individual patient? How might her concerns about the risk of cancer and other side effects of estrogen therapy be answered? Finally, if the patient and physician agree that estrogen treatment is appropriate, what screening tests and follow-up management should be undertaken?

Because of numerous programs proposed by experts on these issues, our goal is to prepare such type of system, which may be the answer of the above-mentioned questions.

Benefits of Hormone Replacement Therapy

(a) Prevention and treatment of osteoporosis –Albright *et al.* (1941) first used orally administered estrogen for osteoporosis and noted an improvement in calcium retention during such therapy. Wallach and Henneman (1959) found that loss of height was arrested during estrogen replacement therapy in 16 to 22 postmenopausal women with established osteoporosis. Later studies showed that estrogen replacement was also effective in the prevention of bone loss by means of stabilization of bone density. Several lines of evidence suggests that the estrogen deficiency is one important cause of postmenopausal (Type-I) osteoporosis Although estrogen receptors, despite many attempts, have not been found previously in human bone, recent convincing studies have shown that cultured human bone cells possess estrogen receptors. Estrogens act primarily to reduce bone turnover, possibly by their ability to diminish the sensitivity of oestoclastic activity to parathyroid hormone. This is thought to be the mechanism by which estrogen therapy decreases the serum calcium concentration in patients with mild primary hyperparathyroidism. Oophorectomy is followed by a rapid decrease of bone mineral density in premenopausal women treated with estrogen therapy. In some retrospective studies women treated with estrogen had a lower incidence of hip or arm fractures than control subjects. This finding was correlated with increased vertebral mineral content in the estrogen treated women in control subjects. Other investigators have found that the risks of hip and forearm fractures are decreased as well by postmenopausal use of estrogen. The Framingham study showed a reduction in risk of hip fractures in women who had taken estrogen within previous 2 years.

At present National Institute of Health Consensus Conference on Osteoporosis, the participants agreed that calcium cannot substitute for estrogen in preventing accelerated bone loss during the first year of menopause, because estrogen deficiency is the primary cause of this phase of bone deterioration (Consensus Conference, 1984). The consensus penal concluded that estrogen replacement therapy should be initiated soon after the onset of menopause as possible. However, because of its side effects, it should be given only to women who are at high risk for osteoporosis (Caucasian race, advanced age, thin habitus, estrogen deficiency, immobilization, weightlessness, extremely strenuous exercise and ammenorrhea, alcoholism, endocrine diseases- hyperthyroidism, Cushing's disease, acromegaly, hypogonadism, hyperparathyroidism), who have no contraindication to use of estrogen, and who will adhere to a program of careful follow-

up (Cuttilon, 1987). Recent reports suggests that extremely strenuous exercise, such as long distance running, may cause decreased level of estrogen, secondary ammenorrhea, and osteopenia that might benefit from estrogen therapy, as does the osteopenia of surgically induced menopause.

The rate of bone loss in lumber spine intensifies to 3% to 6% annually at the time of natural menopause but becomes more gradual after several years (Mezess, 1982). Although few longitudinal studies have been done to assess the effect of estrogen treatment of osteoporosis in older women, available evidence suggests that the beneficial effects of estrogen are greatest during the period of accelerated bone loss immediately after menopause, when type-I (postmenopausal) osteoporosis can occur (Heaney *et al.*, 1986).

(b) Protection against cardiovascular diseases - Estrogen Replacement therapy in postmenopausal women seems to provide a week protective effect against cardiovascular diseases; this effect has been associated with a decrease of serum low-density lipoprotein cholesterol and an increase in high-density lipoprotein cholesterol level. The epidemiologic data relating to this issue are confusing, however and have been well summarized in review by Bush and Barrett-Conner (1985). Stampher and Collogues (1985) concluded that the use of conjugated estrogens without progestins substantially protects women against nonfatal myocardial infarction and fatal coronary artery diseases, but Wilson *et al.* (1985) found that use of similar hormones considerably increases the risks of cardiovascular morbidity, including stroke and myocardial infarction. Ross *et al.* (1987) who recently reviewed eight pertinent case control studies, found that the crude estimates of relative risk of coronary disease associated with estrogen replacement therapy varied from 0.4 to 4.2. After consideration of numerous changes of coronary artery disease risk factor (for example lipids, blood clotting factors, blood pressure, and carbohydrate metabolism) that might occur during estrogen replacement therapy, they concluded that no net change in mortality from ischemic heart disease occur in the patients 50 to 75 years old who receive either high- or moderate- dose estrogen therapy.

Progestins therapy is known as to elevate serum low-density lipoproteins and reduce high-density lipoproteins (Gambrell, 1982) but little information is available to assess the effect of progestins in combination with estrogen on the risk of cardiovascular disease. Measurement of serum lipid before and after 6 months after initiation of estrogen replacement therapy may be advisable.

Other Indication of Hormone Replacement Therapy

1. **Hot flushes-** hot flushes are the commonest menopausal symptoms for which patients seek treatment, and symptoms of perspiration and flushing may persist for more than 5 years. The symptoms are correlated with pulsatile release of luteunizing hormone releasing hormone, which is provoked by estrogen deficiency (Judd *et al.*, 1983), these symptoms are easily corrected with estrogen therapy.
2. **Genitourinary symptoms-** after menopause, atrophic changes of the urogenital epithelium often lead to symptoms of vaginal dryness, burning, itching, dyspareunia, discharge and occasionally bleeding. Patients may also notice dysuria and urinary frequency, even in the absence of urinary infection. Estrogen replacement therapy is effective in restoring the vaginal epithelium to normal and in reversing these symptoms (Barnes and Lobo, 1987).
3. **Skin Changes-** Estrogen is not a panacea for aging, and no available data prove that estrogen therapy confers cosmetic benefits.
4. **Depression-** Mood changes such as irritability and mild depression often accompany menopausal hot flushes and are reversible with estrogen replacement therapy. If sleep disturbances also occur in conjunction with vasomotor instability, estrogen therapy may augment affective and cognitive function by improving sleep pattern (Judd *et al.*, 1983).

Risks from Hormone Replacement Therapy:

1. Uterine cancer: Make *et al.* (1976) showed an increased risk of endometrial carcinoma in postmenopausal women treated with orally administered conjugated estrogens. The addition of progestogen reduces the incidence of uterine cancer.

2. Thromboembolism: Although thromboembolic phenomenon have been associated with the use of oral contraceptive agents and clinical studies to date have shown no increased risk of thromboembolism in postmenopausal women during orally administered estrogen replacement therapy. Recent study of transdermally administered estrogen showed no change in clotting factor and other liver proteins (rennin substrate, thyroxine-binding, cortisol-binding, and sex hormone binding globulins), which are known to be increased by orally administered estrogens.

3. Hypertension: rennin substrate is increased during oral estrogen therapy, and this finding may be associated with increases in angiotensin II and aldosterone; however, these changes may be of no clinical consequence. Of note, transdermal estrogen therapy is not associated with change in circulating rennin substrate.

(B) CONTRACEPTION:

Like other animals, man has a natural desire to reproduce his kind. However, unlike animals, he alone can control the reproduction at will and plan his family. Man's great reproductive potential and greatly increased survival rates in recent years have posed a major problem of population growth with far reaching consequences. According to the United Nations estimate, at the present rate of growth, the world population is expected to double in 35 years and it would increase eight-fold in a century. Nearly 4000 babies are born every minutes of every day in this world.

If the world resources are considered, this forecasts a disaster for human race, unless the fertility is controlled. As for India, the equivalent of the entire population of Australia is added every year! Fertility control is very essential for maintaining satisfactory standards of living and for raising the existing standards in developing countries. Furthermore, it is equally important for family planning, which is defined as "the limitation and spacing births in the interests of the mother, the child and the rest of the family". The problem is essentially interdisciplinary and can only be solved by the concerted efforts by biologists, sociologists and educationists; the use of pharmaceutical agents for this purpose is but one aspect of the whole programme.

Political, cultural and research-cost barriers have enormously complicated the development of contraceptive agents in modern times. The reviews by Djerassi, inventor of norethindrone and by Lednicer are important reading. The most notable achievement in chemical contraception came in the late 1950s and early 1960s with the development of oral contraceptives agent "the pill". Since then, a variety of contraceptive products have been introduced, including hormone-releasing intrauterine device, polymer implants, injectable formulations and a transdermal patch. The failure rate of different contraceptive methods and marketed products used for contraception are given in table-1.

Table 1.1: Failure rate of different contraceptives methods

S. No.	Methods	Pregnancies/100 women year
1	Tubal ligation	0.5
2	Vasectomy	0.15
3	Norplant	0.05
4	Implanon	0.05
5	Depoprovera-injection	0.3
6	MPA/E2C injection	<1
7	Combination oral pill	0.1-3
8	Progestin only minipill (oral)	0.5-3
9	Progestrasert IUD	1.5-2
10	Copper T IUD	0.6-0.8
	Levonorgestrel releasing IUD	0.1
11	Nuvaring	1-2
12	Transdermal combination contraceptives	1
13	Diaphram with spermicides	6-20
14	Condom (Female)	5-21
15	Condom (Male)	3-14
16	Withdrawal	4-19
17	Spermicides	6-26
18	Periodic abstinence	9-25
19	No contraceptives	85

Table 1.2: Comparison of steroid contraceptive regimens

S. No.	Brand	Drug	Dosage Cycle
1	**Combination-Monophasic**		
	Levlin	Levonorgestrel 0.15 mg	Ethinylestradiol, 30µg
	Nordette	Levonorgestrel 0.15 mg	Ethinylestradiol, 30µg
2	**Combination-Biphasic**		
	Jenset-28	7days: Norethindrone, 0.5 mg	Ethinylestradiol, 35µg
		14 days: Norethindrone, 1 mg	Ethinylestradiol, 35µg
	Ortho Novum	10days:Norethindrone,0.5 mg	Ethinylestradiol, 35µg
		11 days: Norethindrone, 1 mg	Ethinylestradiol, 35µg
3	**Combination-Triphasic**		
	Ortho Novum 7/7/7	7 days: Norethindrone, 0.5 mg	Ethinylestradiol, 35µg
		7 days: Norethindrone, 0.75 mg	Ethinylestradiol, 35µg
		7 days:Norethindrone, 1 mg	Ethinylestradiol, 35µg
	Estrostep	5 days: Norethindrone acetate, 1 mg	Ethinylestradiol, 25µg
		7 days:Norethindrone acetate, 1 mg	Ethinylestradiol, 30µg
		9 days:Norethindrone acetate, 1 mg	Ethinylestradiol, 35µg
4	**Progestin Only**		
	Micronor	Norethindrone, 0.35 mg	----
	Overette	Norgestrel, 0.075 mg	----
5	**Injectable depot Hormonal Contraceptives**		
	Depoprovera	Medroxyprogesterone acetate alone	150/month 150 mg every 3 months
	Lunelle	Medroxy progesterone acetate 25 mg + estradiol cypionate 5 mg/ 0.5ml	0.5 ml IM injection once in deltoid, gluteus mamimus, or anterior thigh every 28 to 30 days
6	**Transdermal Contraceptives Patch**		
	Ortho-Evra	Norelgestromin 6 mg, ethynilestradiol 0.075 mg / 24 hrs	One patch each week for 3 weeks. 1 week no patch.
7	**Hormone releasing Implants, IUD, and Vaginal rings**		
	Progestasert	Progesterone releasing IUD	38 mg dose in IUD lasts 1 year
	Mirena	Levonorgestrel releasing IUD	52-mg dose in LRIS provides contraception for upto 5 years
	Norplant	6 silastic capsules with 36 mg levonorgestrel: all capsules are inserted subdermally in the middle upper arm	Contraception efficacy lasts up to 5 years if the implants are not removed
	Implanon	One polymeric rod with 68 mg etonorgestrel, released at a rate –40 µg/day	Contraception efficacy lasts up to 3 years if the implants are not removed
	Nuva Ring	11.7 mg etonorgestrel, 2.7 mg ethinylestradiol in a flexible, vaginal ring	Vaginal ring is inserted for 3 weeks duration, then 1 week off before insertion of new ring
8	**Emergency Contraceptives**		
	Plan B	0.75 mg Levonorgestrel	The first dose (1 tablet) should be taken as soon as possible within 72 hrs of intercourse, the second dose must be taken 12 hrs later

In the past, estrogens in most estrogen replacement therapy have been administered orally. The high concentration of estrogen in the portal circulation after oral administration, however, leads to increased hepatic production of estrogen dependent proteins and bile changes that reportedly account for several of the complications of the estrogen therapy including cholethiasis, thrombophlebitis, and pulmonary embolism. Transdermal administration presumably can prevent these complications and has the theoretic advantage of continuous rather than bolus absorption into the circulation.

TRANSDERMAL DRUG DELIVERY

Transdermal drug delivery systems are specifically designed to obtain systemic blood levels and have been used in U.S. since 1950s: only recently have these systems been redefined. Transdermal permeation or percutaneous absorption can be defined as the passage of substance, such as a drug from the outside of the skin through its various layers into the bloodstream. Any time if there is systemic accumulation of a drug, unwanted side effects or toxic effects can occur. Certainly, each dosage form has its unique place in medicine, but some attributes of transdermal delivery system provide distinct advantage over the traditional methods of attaining systemic level of drugs. Clearly has listed important advantages and disadvantages of the transdermal delivery systems. The advantages of transdermal delivery system are that it,

1. Does not have GI distress or other physiologic contraindications of the oral route
2. Provide adequate absorption of drugs with some oral estrogen promptly
3. Increases patient's compliance.
4. Avoid first pass effect
5. Allows effective use of drugs with short biological half lives
6. Allows administration of drug with narrow therapeutic window.
7. Provide controlled plasma levels of potent drugs and
8. Interrupts drug input promptly when toxicity occur.

Disadvantages of this system include

1. Drugs that require high blood levels can not be administered
2. Adhesive may not adhere well to all skin
3. Drug or drug formulation may cause skin irritation or sensitization
4. It is uncomfortable to wear and
5. System may not be economical

In the development of transdermal delivery systems, a series of interrelated elements must be taken into consideration. These factors can be classified into five basic areas: bioactivity of drugs, skin characteristics, formulation, adhesion, and system design. The transport of drugs through skin is complex since many factors influence their permeation. To simplify it somewhat, one can look at:

1. Skin structure and its properties
2. The penetrating molecule and physical-chemical relationship to skin and delivery platform.
3. The platform or delivery system carrying the penetrant, and
4. The combination of skin, the penetrant, and the delivery system as a whole (Juliano and Chein, 1987).

Theoretical Advantages of Transdermal Route:

It is usual to contrast the percutaneous route with oral delivery as the latter provides the most popular way for delivering medicaments in general. The transdermal input of a drug would estimate several variables that make gastrointestinal absorption a problem. These factors include the dramatic changes in pH as the molecules moves from gastric acid with a pH as low as 1 to the intestine with a pH of up to 8. Other variables that may be obviated include gastric emptying; intestinal motility and transit times, the operation of human and bacterial enzymes, and the influence of food on drug absorption.

Via skin, the drug enters the systemic circulation without hepatic first passing into the portal system and traversing the liver. The route therefore avoids the first-pass phenomenon by which the liver can significantly reduce the amount of intact agent that passes into the systemic circulation. Additionally, the medicament avoids enzymes that are present in the gut wall. However, as emphasized earlier, the skin itself possesses a metabolic capability.

For a correctly formulated product, the percutaneous input of a drug can control administered drug and thus limit pharmacologic action; indeed the corresponding oral or injectable formulation may well elicit several effects that include toxic reactions. Patient's compliances may be helped by the continuity of input of drugs with short half-lives.

Transdermal administration under suitable rate control could minimize pulse entry into the blood stream. Undesirable side effects being particularly associated with peak plasma levels. However, a more difficult matter is to deliberately provide a controlled on/ off action because intact skin membranes are intrinsically slow response system with prolonged lag time, at least when shunt diffusion via the appendages is neglible. Percutaneous administration can be valuable for drugs with low therapeutic indices e.g. those for which the toxic concentration in the plasma is near the clinical level.

Some investigators claim that it would be easy to terminate therapy by simple removal of a topical device so as to interrupt medicament delivery. However, the stratum corneum would continue to deliver molecules to the viable tissues for some time after device removal, at a declining rate as governed by the properties of the drug reservoir. This is another consequences of the long response times of horny layer membrane.

Optimization of Percutaneous Absorption:

When formulators develop dermatologic preparations for optimum bioavailability, they employ two main methods of approach either singly or combined. The first scheme formulates the vehicle or device so that the drug has the maximum tendency to have the base and to partition into the skin. The formulator does not intend that the vehicle component should affect the physiochemical properties of the stratum corneum. Thus the vehicle design promotes the drug release by simply optimizing chemical potential of the medicament. However, even the most innocuous of vehicles tends to change the nature of stratum corneum if only by hydrating it. The alternative strategy incorporates materials known as **penetration enhancers** into the formulation. These are chemicals that enters the skin dynamically and reversibly altering it to promote the penetration of drugs. The desirable attributes of such enhancers have been listed by Berry *et al.* (1987) and they include the following:

1. They should be pharmacologically inert, interacting with no receptors in the skin or in the body generally.
2. The enhancer should be neither toxic, irritating nor allergic.
3. The onset of enhancer activity and the duration of effects should be predictable, controllable and suitable.

4. The skin should show an immediate and full recovery for its normal barrier property when the enhancers leave the tissue.
5. The accelerant should promote penetration into the skin without developing significant problems of loss of body fluids, electrolytes or other endogenous materials.
6. The chemical should be compatible with a wide range of drugs and pharmaceutical adjuvants.
7. Where appropriate, the substance should be suitable solvent for the drugs.
8. For traditional formulations, the material should spread well on the skin and it should have a suitable skin feel.
9. The chemical should be suitable for formulate into creams, ointments, gels, lotions, suspensions, aerosols, skin adhesives, and delivery device.
10. It should be odorless, tasteless, colorless, and relatively inexpensive (Woodford and Barry, 1984).

Table 1.3: Marketed Tablets and transdermal patches of combined progestins/ estrogen hormones

S. No.	Brand	Progestins	Estrogen
1	Prempro	Medroxyprogesterone acetate 2.5-5.0 mg	Conjugated estrogen, 0.625 mg
2	Premphase	Medroxyprogesterone acetate 5.0 mg	Conjugated estrogen, 0.625 mg
3	Femhrt	Norethindrone acetate, 1 mg	Ethinyl estradiol, 5 μg
4	Activella	Norethindrone acetate, 0.5 mg	Estradiol, 1 mg
5	Ortho-Prefest	Norgestimate, 0.09 mg	Estradiol, 1 mg
6	CombiPatch	Norethindrone acetate, 0.14 or 0.25mg	Estradiol, 50 μg

Table 1.4: Marketed transdermal patches of different drugs

Drugs	Trade name	Type of device	Indication
Scopolamine (Hyoscine)	Transderm-Scop Kimite Patch	Reservoir	Motion sickness
Nitroglycerine	Transderm-Nitro Deponit Nitro-Dur Nitrodisc NTS	Reservoir Mixed monolithic Reservoir Monolithic Monolithic Monolithic Monolithic	Angina Angina Angina Angina Angina Angina Angina
Isosorbide-dinitrate	Frandol Tape	Monolithic	Angina
Clonidine	Catapress TTS	Reservoir	Hypertension
Estradiol	Estraderm	Reservoir and ethanol enhancer	Hormone treatment
Estradiol esters	--	+	Hormone treatment
Testosterone	--	+	Hormone treatment
Timolol	--	+	Cardiovascular
Propanolol	--	+	Cardiovascular
Fentanyl	Duragesic	--	Opoid Analgesic
Glycol Salicylate	--	+	Analgesic
Methyl Salicylate	--	+	Analgesic
Chlorpheniramine	--	+	Antihistamines
Diphenhydramine	Zenol	--	Antihistamines
Physostigmine	--	+	Cholenergic
Insulin	---	+	Diabetes
Nicotine	--	+	Aid to smoking Cessation
Albuterol	--	+	Bronchidialator
Piroxicam	--	+	Arthritis
Ketorolac	--	+	Non Narcotic analgesics
Flubiprofen	Zepolas	--	Anti-inflammatory
Indomethacin	Indomethin	--	Anti-inflammatory
Bufaralol	--	+	Angina, hypertension
Bupranolol	--	+	Angina, hypertension, Anti-glaucoma agent

The transdermal drug delivery systems for estrogens and progestins covers skin structure and absorption of chemical agents by diffusion and partition, permeability and use of enhancers to speed absorption, choice of drugs for transdermal contraceptives, animal and clinical work with estradiol, ethinylestradiol and levonorgestrel, use of prodrug derivatives, types of transderm delivery systems, metabolism of these drugs by skin and skin flora, and cutaneous side effects, all illustrate graphically and mathematically.

NIOSOMES AS CARRIER FOR TRANSDERMAL DRUG DELIVERY:

Niosomes appears to have application in topical and transdermal product both containing hydrophobic and hydrophilic drugs. Beside the niosomes have also been used to encapsulated, lidocaine (Vanhal *et al.*, 1996), estradiol (Vanhal *et al.*, 1996), cyclosporin (Dowton *et al.*, 1993), erythromycin (Jayaraman *et al.*, 1996), alpha interpheron (Niemac *et al.*, 1995), plasmid DNA for human interleukin-1 receptor (Niemic *et al.*, 1997), for topical and transdermal delivery.

Junginger *et al.* (1991) have observed small (100 um) vesicular structures between the first and second layer of human cornecytes on 48 h incubation with niosomes prepared from dodecyl alcohol, polyoxyethylene ether and cholesterol. Penetration of niosomes of this upper layer appears plausible as these layers are only loosely packed. However, the same study reports the presence of vesicular structures in the deeper layers seemingly inaccessible was on the skin and concludes that there was a reorganization of the niosome membrane into individual monomers which on arriving at these deeper layers reformed into niosomes (Junginger *et al.*, 1991).

Vanhal *et al.* (1996b) have performed in-vitro studies on the transdermal penetration of oestradiol using high phase transition temperature of sucrose ester niosomes or $C_{18}EO_7$ niosomes and low phase transition temperature $C_{12}EO_7$ niosomes. They found that $C_{12}EO_7$ niosomes are better transdermal carriers. The higher flexibility of these bilayers is said to be responsible for this improved transdermal penetration. Reducing the cholesterol content of these niosomes also increases the transdermal delivery of oestradiol.

The intracellular route is the main route of vesicle penetration across the skin (Schatzlein and Ceve, 1993, Schatzlein and Ceve, 1998, Kuijk-Meuwissen *et al.*,1998). Ultraflexible vesicles penetrate along irregularities between the intracellular lipid

lamellae and adjacent cornecyte envelop. The combination of molecules with suitably differing molecular shapes can render a membrane flexible. The flexible membrane accommodates stress induced curvature changes and the vesicle shape changes easily. Consequently such vesicles require significantly less deformation energy to pass through the small pores than rigid membrane. Sufficiently elastic vesicles can penetrate into deeper layers but rigid vesicles remain restricted to the loosely packed upper layer. Bergh *et al.* (1988) prepared flexible liquid state niosomes from polyoxy ethylene laurate ester, sucrose laurate ester and cholesterol sulphate and studied the penetration behavior of these niosomes. They found that the flexible niosomes could penetrate and permeate through skin.

Hu *et al.* (1994) studies the delivery of Cyclosporin A in the number of formulation after topical application in hairless mouse model. The migration of Cyclosporin A from Cyclosporin A glyceryl dilaurate/$C_{16}EO_{10}$/ Cholesterol niosomes into the deeper strata has also been studied in-vitro and it was found that the factor such as dosing volumes produce an increased uptake of the drug into deeper skin strata (Niemec *et al.*, 1994). Based on above studies, it does appear that transdermal drug delivery with niosomes appears promising for both hydrophobic and amphiphilic drug molecules and would require higher doses to be applied by entrapping the drug in niosomes prepared from low phase transition temperature.

Various types of vesicles skin interactions, which were suggested by scientists, also depend on different factors, which are as follows:

Nature of drug: Lipoidal drug prefer the lipid barrier of the skin layer while the hydrophilic drug in the vesicular bilayers, partition and reach into follicular sweat ducts, which contain lipid barrier.

Dehydration of vesicles: Surfactants are responsible for rate of hydration of vesicular bilayer and control the extent and rate of dehydration and transfer of drugs into skin which in turn control the rate of transfer of both hydrophilic and hydrophobic drugs. For effective transdermal vesicular system, vesicles remain in equilibrium dehydration condition and after complete dehydration condition and, transition from liquid to gel state ceased the transfer of drug into the skin (Touitou *et al.*, 1994; Weiner *et al.*, 1994 and Plessis *et al.*, 1994).

Size and composition of vesicles: Small vesicles with a high-cholesterol ratio have shown significantly greater accumulation in the skin than the vesicles with low lipid-cholesterol. Small vesicles exhibit lower skin penetration, higher accumulation in the skin and longer lag time than larger vesicles.

Biophysical factors: As the isoelectric point of the stratum corneum is about pH 3-4, the surface of the skin bears a negative charge at natural pH 5-6. In this way, the skin acts as a capacitor with a surface that attracts cations. The cations can be driven into the skin by anode and anions by the cathode. Upon ultrasound irradiation, cavitation in the tissue fluids occurs and this may be the reason for an altered chemical reactivity and cleavage of macromolecules and therefore changed membrane properties (Fisher, 1992).

Vesicles for contraceptives:

Liposomes/niosomes have shown great potential as a topical drug delivery system. The first report on topical application of liposomally encapsulated steroid drug was presented by Mezie and Gulshekgharan (1979). The studied triamcinolone and triamcinolone acetate gel, and observed that the concentration of drug was five times higher in epidermis and four times higher in dermis than controlled gel. Topical product formulators have tried liposomal encapsulation on large variety of drugs commonly used in conventional, topical dosage form e.g. topical corticosteroids, antibiotics, anesthetics agents, preservatives, enzymes and retinoids. Switzerlands was the first country to approve a topical liposomal formulation.

Vanhal *et al.* (1993) reported in-vitro diffusion of niosomes encapsulated estradiol through human stratum corneum. Recently prototype liposomal delivery system, which consisted of thin agarose gel containing progesterone as a model drug has been reported.

Knepp *et al.* (1990) investigated the transfer mechanism of progestins in more detail by comparing the transdermal flux of progesterone and steroid in liposome vesicle. The liposome vesicle exhibited zero order kinetics of transdermal flux for over 24 hrs. Furthermore, there were no appreciable differences in transdermal flux of progesterone from liposome in suspension or progesterone containing liposome immobilized in a gel.

Victoria *et al.* (1990) reported the evaluation of prototype liposomal delivery system, which consisted of thin agarose gel dispersed throughout progesterone associated with multilamellar liposomes. They observed that the transdermal delivery of progesterone from acyl chain phospholipids was of an order of magnitude lower than cis-unsaturetd phospholipid formulations.

Liposomal based intravaginal delivery system for contraceptive use was studied by Sahu and Jain (1997) containg progesterone loaded liposomes. Calculated quantity of liposomal pellet was mixed thoroughly into hydrogel. This when administered intravaginally improved contraceptive action.

Vora and Jain (1996) reported absorption of proniosomal vesicles bearing levonorgestrel through transdermal route for contraception. These proniosomal gels converted into niosomes after application to the skin due to hydration.

PRONIOSOMES:

Niosomes are unilamellar or multilamellar vesicles formed from synthetic non-ionic surfactants offering an alternative to liposome as drug carriers (Uchegbu and Vyas, 1998). An increasing number of non-ionic surfactants have been found to form vesicles, capable of entrapping hydrophilic and hydrophobic solutes (Yoshioka *et al.*, 1994). Niosomes are important from technical viewpoint as that possess greater stability and avoid some disadvantages associated with liposomes such as variable purity of phospholipids and high cost (Vora *et al.*, 1998). Another advantage of niosome is the development of a simple practical method for routine and large scale production without the use of pharmaceutically unacceptable solvents. Proniosomes offer a versatile vesicle delivery concept with potential for delivery of drugs via transdermal route (Vora *et al.*, 1998, Huang *et al.*, 1997). This would be possible proniosomes form niosomes upon hydration with water from skin following topical application under occlusive conditions. The aim of this study is to examine the feasibility of proniosomes as a transdermal drug delivery system of Ethinylestradiol and Levonorgestrel.

Proniosomes are the formulations of surfactant-coated carrier, which may be measured out as needed and rehydrated by brief agitation in hot water (Hu and Rhodes, 1999). These proniosomes minimizes problems of physical stability of niosomes such as aggregation, fusion and leaking and provide additional convenience in transportation, distribution, leakage and dosing. Proniosomes are normally prepared by spraying surfactant in organic solvent and sorbitol powder and then evaporating the solvent. Because of sorbitol carrier is soluble in the organic solvent, it is necessary to repeat the process when the desired surfactant load has been achieved. Surfactant coating on the carrier comes out to be very thin and hydration of this coating allows multi-lamellar vesicle to form.

Drugs containing proniosomes derived niosomes should be prepared in manner analogues to that used for conventional niosomes, by adding drug to the surfactant mixture prior to spraying the solution on to sorbitol, or by addition drug to the aqueous solution used to dissolve/ hydrate the proniosomes. Proniosomes derived niosomes are superior to conventional niosomes in convenience of storage, transport, and dosing. Stability studies are still in progress, but it is expected that the dry proniosomes preparation will be more stable than a pre manufactured suspension. In release studies, proniosomes appear to be equivalent to conventional niosomes (Rhodes and Hu, 1999). Size distributions of proniosomes derived niosomes are somewhat better than those conventional niosomes, so the release performance in more critical cases turns out to be superior. Proniosomes are dry powder, which makes richer processing and packaging possible. The powder forms derived niosomes are better than conventional niosomes. Niosomes appears to have application in providing optimal flexibility, unit dosing, in which the proniosomes powder provided in capsule could be beneficial. A slurry method is developed to produce proniosomes using cyclodextrin as a carrier. The time required to produce proniosomes by this method is independent of the ratio of surfactant solution to carrier material. In the slurry method, the entire volume of surfactant solution is added to malto-dextrin powder in a rotary evaporator and vacuum applied until the powder appears to be dry and free flowing.

There are different types of proniosomes e.g. Free flowing proniosomes, Mixed miceller proniosomes, and Mesophasic proniosomes.

(a) Free flowing proniosomes can be prepared by coating lipid on an inert material precisely sorbitol bed was prepared by warming evacuated rotatary flask. On this bed, lipids alongwith drug were coated by feeding methanolic solution of lipid and drug. After complete evaporation, the product was sieved through 950-micron stainless steel mesh. The product was free flowing granular material, e.g. Amphotericin-B provesicles (Pyane *et al.*, 1986), propanolol (Byung *et al.*, 1995), 5-flurouracil, ibuprofen, indomethacin. A variety of materials have been investigated for suitability in the manufacturing of proniosomes. Desirable characteristics include good water solubility (for ease of proniosomes hydration), poor solubility in chloroform and/ or methanol for ease of processing, and suitability for intravenous use. Materials tested include glucose, fructose, lactose, and sorbitol. The suitability of various particle sizes lots of sorbitol for proniosomes manufacture was examined initially. In general all materials in the range of

125-500μm were suitable for manufacture when a constant weight ratio of liposomal components to sorbitol was maintained and had no effect on subsequent liposomal size. Material in particle size <125μm was unsuitable due to excessive agglomeration during manufacture.

(b) Mixed miceller proniosomes: mixed micelles of phosphatidyl choline and bile salt act as proniosomes. Lipophilic drugs can be solubilized using mixed miceller system. The proniosomes were obtained on removal of solvent, from alcoholic stock solution of lecithin and bile salts and drug on reconstitution using buffer solution vesicles were obtained. The removal of solvent phase and bile contents affect the miceller size. With high removal of solvent phase and low bile content-formed large micelle, while low removal of solvent phase with high bile content-formed liquid crystalline aggregates e.g. Treponoside proniosomes (Onyuksel and Son, 1995).

(c) Coacervation phase separation: This method is similar to the coacervation phase separation technique. Lipid/surfactants mixture is dissolved in warm alcohol, which acts as a good solvent. The buffer is added which acts as non-solvent. Using temperature change forms Lameller liquid crystals. In case of lecithin, non-ionic surfactants and alcohols, the mixture again heated on water bath and then it is allowed to cool. The resultant formulation has stalked bilayer structure.

Perret *et al.* (1991) first developed this method. Ishii *et al.* (1995) prepared proniosomes and studied the effect of alcohol on particle size of niosomes formed after dilution. The proniosomes offer many advantages.

1. They increase the stability of vesicles both chemical well as physical.
2. Proniosomes can be used as carrier system for targeting the drugs.
3. The solubilization of drug(s) can be achieved using proniosomal system.
4. Proniosomes itself can serve as sustained release drug delivery system.

LITERATURE SURVEY

Blumel *et al.* (2003) studied the effect of transdermal estrogens (patch containing 50ug) on endothelial function in postmenopausal women. They found that transdermal estrogen might improve endothelial function.

Creasy *et al.* (2003) studied the effect of transdermal contraceptive patch delivering norelgestromin and ethinylestradiol on lipid profile. The lipid changes seen with the contraceptive patch were consistent with those of oral contraceptives containing norgestimate and ethinylestradiol

Sigat Briggate (2003) developed a new contraceptive patch Ortho Evra approved for the prevention of pregnancy. In two clinical trials, compliance was greater with patch than with oral contraceptives.

Vincent *et al.* (2003) studied the effect of estrogen replacement therapy on the secretion of parathyroid hormone and found that, in elderly postmenopausal women, estrogen does not have significant direct effect on PTH secretion. They have pointed the importance of the actions of estrogen on intestinal and renal calcium handling as the major mechanism for its effect on modulating calcium homeostasis and indirectly, PTH secretion.

Ibarra *et al.* (2002) evaluated skin irritation and adhesion of Estradot and Climara patches in healthy postmenopausal women and found that estradot produced less skin irritation and better adherence.

Mueck *et al.* (2003) suggested that women who continued to smoke despite all warnings should only be treated via the transdermal route. They have also suggested that oral contraceptives are contraindicated in elderly women smoker but hormone replacement therapy may be given to such women.

Costa *et al.* (2003) evaluated in-vitro release profiles of 13 patches of estradiol (from five marketed products) by using paddle-over-disk method. They have also tested several release models like zero order, first order, Higuchi, Weibull, Korsmeyer-Pappes, and Makoid Banker. The release profile showed that the drug was released at constant rate from three patches while it was not constant from other patches diminished with the squire root of time (Higuchi Model).

Basurto *et al.* (2002) studied the serum level of cortisol, insulin, lipoproteins and leptin by using specific assay methods before and after 3 months of transdermal estradiol therapy. They found that this therapy induced a decrease in circulating cortisol, insulin, triglycerides and low-density cholesterol to normal values which may have a beneficial effect in menopausal women.

Fauth *et al.* (2002) studied tensile strength and peel adhesion of seven branded transdermal patches (Alora, Cutanum, Estraderm MX 50, Estraderm TTS 50, Fem7-50, Monorest, and Oesclim). For the branded transdermal patches no correlation was found between young modules and both the peel force on stainless steel and the skin adhesion.

Toole *et al.* (2002) evaluated two-estrogen patches for skin irritation and sensitization and found that Estradot demonstrated lesser skin irritation, superior adhesion and a lower rate of patch loss as compared to Monorest.

Barry *et al.* (2002) studied drug delivery in skin, employing two human skin membranes to differentiate the shunt route delivery from bulk transepidermal membrane and compared its delivery through a sandwich of stratum corneum and epidermis.

Araujo *et al.* (2002) compared the effect of oral and transdermal estrogen replacement therapy on lipid and glucose metabolism in postmenopausal women with diabetic mellitus type 2 and they have reported that there was no harmful effect on glucose metabolism in type 2 diabetic postmenopausal women .

Harrison *et al.* (2002) evaluated the bioequivalence of two 7-day 17 beta-estradiol transdermal systems namely Estraderm and Climara, From this study they have concluded that bioequivalance at one anatomical site is not indicative of bioequivalance at another site of patch application.

Jamic (1993) reviewed benefits of high dose of progestational contraceptives like norpregnanes, promegestone, nomegestrol acetate, its antioestrogenic effect and its overall absence of metabolic effects. In conclusion high dose progestestational contraceptives produced excellent metabolic results and clinical manifestations of hyperestrogeny.

Notelovitz *et al.* (2002) studied the effectiveness of Alora estradiol matrix type transdermal delivery system and reported that by applying the patch twice weekly improved lumber bone mineral density in healthy postmenopausal women, with the benefits of treatment evident by one year. The lowest effective dose was 0.25 mg/day.

Kaplan *et al.* (2002) evaluated the effects of continuous and cyclic 17-beta estradiol and norethindrone acetate combination on platelets aggregation. They have concluded that continuously oral or cyclically transdermal and sequentially does not affect platelets aggregation in postmenopausal women.

Lo dico *et al.* (2002) studied the effect of hormone replacement therapy in postmenopausal women on endometrium histology and bleeding pattern and found that endometrial pattern was secretary in 60 cases, proliferative in 5 and atrophic in 22 but the endometrial histology characteristics haven't shown any influence on bleeding pattern.

Funke *et al.* (2002) studied the effect penetration enhancers on in-vitro release and transdermal fluxes of a highly lipophilic drug from poly-acrylate based matrix TDS and they studies suggested that the highly lipophilic antiestrogen can be administered transdermally by penetrating the skin with fluid permeation enhancer in combination with propylene glycol-lauric acid (9+1).

Corrazza *et al.* (2002) presented a case study of allergic contact dermatitis from transdermal estradiol and systemic contact dermatitis from oral estradiol and they suggested that positive test with estradiol led to the diagnosis of type IV allergic dermatitis due to transdermal estradiol and a gel containing estradiol. Systemic contact dermatitis due to oral estradiol was also diagnosed but allergic contact dermatitis from estradiol is extremely rare.

Likis *et al.* (2002) reviewed the contraception methods of formulations containing estrogen with newer combined contraceptives product including the injection, vaginal ring, and patch.

Khassoggi (2002) reported that there is overwhelming evidence that hormone replacement therapy improves the quality of life and reduces the morbidity and mortality by reversing the metabolic and pathological changes induced by the menopause. The benefits of hormone replacement therapy out weigh any increased risk of venous thromboembolism or breast, ovarian and endometrial cancers.

Paoletti *et al.* (2002) studied oral or transdermal hormone replacement therapy containing 17-beta estradiol and levonorgestrel seems to exert beneficial effects on the main postmenopausal symptoms without negative interferences on the endometrium. The bone re-absorption and the LDL cholesterol level significantly decrease only in-group A and C, group B (estradiol+levonorgestrel) bone re-absorption was significantly increased.

Williams *et al.* (2002) studied the effects of exercise on serum level of estradiol (E2), estrone(E1), cartisol, growth hormone, insulin, glucose and lactate and they found that growth hormone, Cortisol and , insulin all changed significantly to the 45 minutes exercise bouts, but no difference were observed between oral micronised estradiol and transdermal estradiol. The estradiol level was increased significantly after the transdermal estradiol 45 minutes exercise bouts. This change did not occur during the oral estradiol exercise bouts.

Sturdee *et al.* (2002) studied the endometrial safety of a transdermal estradiol-levonorgestrel combination. The endometrium biopsy was done before and after one year of treatment. Estradiol and sequential levonorgestrel administered in a 7-day transdermal matrix patch for one year provided endometrium protection.

Waddell *et al.* (2002) studied distribution and metabolism of topically applied progesterone in rat skin. Concentration of lipid and water-soluble metabolites of progesterone were also measured in plasma, urine, and selected tissues (uterus, liver, kidney, salivary gland) 3 h after its application. Plasma progesterone increased progressively until 90 minutes then remain stable. Plasma levels of progesterone were high and increased only slightly thereafter. Water-soluble metabolites were detectable in plasma at 15 minutes, whereas lipid soluble metabolites became apparent only by 60 minutes, then progressively increased upto 180 minutes. The tissue plasma concentration was higher in uterus and lungs.

Van de Weijer *et al.* (2002) studied the effects of estradiol and levonorgestrel delivered transdermally by 7 day patches in a sequential combined regimen. It showed very good cyclic control that was well accepted by postmenopausal women.

Rohr Uwe *et al.* (2002) studied the removal and reapplication of 17 beta estradiol transdermal patch in post menopausal women. They found that the TDS yielded sustained estradiol plasma level in postmenopausal women in the initial application period. In long term dosing, there was no significant drop in estradiol level after patch removal.

Riginster *et al.* (2000) developed a matrix 17 beta estradiol transdermal patch incorporating lauric acid to improve estradiol skin absorption and suggested that the matrix patches incorporating lauric acid led to estradiol plasma levels more stable than with the reference matrix and reservoir patches, and greater than those with reference matrix patch.

Akerblom *et al.* (1998) studied the influence of hormone replacement therapy on electrocardiogram pattern in hypercholesterolemic women and to assess the possible influence of female sex hormone. The study revealed a higher prevalence of pathological ECG changes in postmenopausal women who had hypercholesterolemia than in normocholesterolemic women. These finding supported the idea that hyperlipidemia contributes to the overall increase in cardiovascular diseases as this also associated with ECG changes. Transdermal patch containing estradiol and medroxy-progesterone acetate has beneficial effect inrevesing the process of atherosclerosis as well as improved the ECG pattern.

Von Holst Thomas *et al.* (2002) investigated the efficacy and tolerability of a new 7-day sequential estradiol and levonorgestrel patch (Fem7) verses placebo as hormone replacement therapy in postmenopausal women. The study suggested that a new 7 day

sequential estradiol and levonorgestrel patch (Fem7) was well tolerated and provided rapid and effective relief of menopausal symptoms as compared with placebo. The addition of low dose levonorgestrel did not influence the beneficial effects of estradiol.

Zieman *et al.* (2002) studied the contraceptive efficacy and cyclic control with Ortho Evra / Evra transdermal system and found that the overall annual pregnancy was 0.8% and the method failure possibility was 0.6%. The efficacy of patch was high and similar across age racial groups. Among women <90kg, contraceptive failure was low and uniformly distributed across the range of body weights. In women >90kg contraceptive failure may be increased.

Dittrich *et al.* (2002) studied three transdermal norelgestromine / ethinylestradiol contraceptives doses 10, 15, 20-cm patches, OrthoEvra/Evra-20cm (2), Ortho-Cyclen / Cilest. They found that, Ortho-Evra / Evra-20cm (2) provided suppression cycle control and safety similar to that of Ortho-Cyclen / Cilest with significantly better compliance.

Creasy *et al.* (2001) performed a study on once-weekly OrthoEvra / Evra contraceptive patch. The patch deliverd norelgestromin, 150microg, and ethinylestradiol, 20 microg, daily to the systemic circulation. The contraceptive patch provided ovulation suppression and cycle control similar to that of oral norgestimate 250 microg /EE 35microg. The patch was not associated with phototoxicity or photo allergy. The contraceptive patch is the only noninvasive, weekly birth control method that a woman can self-administer.

Colacurci *et al.* (2001) studied the effect of different types of hormone replacement therapy on mammographic density in postmenopausal women. They found that HRT might cause an increase of mammographic density. The frequency of the density increase is related to the type of HRT and a replacement therapy including a progestin, especially in continuous combination with estrogen, leads to more evident mammographic changes. Tibolone does not significantly affect mammographic density.

Sator *et al.* (2001) measured the thickness by high frequency ultrasound to objectify the effect of HRT in the menopause. This study showed the influence of three different HRT regimens from comparison of changes in skin thickness. The study showed that HRT lead to the increase of skin thickness, which can be demonstrated by a high frequency ultrasound.

Vantinnen *et al.* (2001) studied the in-vivo transdermal absorption of the isoflavonone, diadzein and genistein, applied on the skin in olive oil. The concentration

of isoflavonone and their metabolite were monitored in plasma and urine by GC-MS methods. They found that diadzein and genistein are captured in the skin following repeated transdermal application, diadzein and genistein, applied.

Soares *et al.* (2001) studied the efficacy of estradiol for the treatment of depressive order in perimenopausal women. Previous studies suggest that estrogen improves somatic and mild depressive symptoms experienced by perimenopausal. In this study the efficacy of 17-beta estradiol for the treatment of clinically significant depressive disorders in endocrinology confirmed perimenopausal women was investigated. Remission of depression was observed in 17 (68%) women treated with 17-beta estradiol compared with 5 (20%) in the placebo group. Transdermal estradiol replacement has been proved to be effective treatment of depression for perimenopausal women.

Vongpatanasin *et al.* (2001) applied transdermal patches for estrogen replacement therapy on female, which decreased sympathetic activity in postmenopausal women. Menopause causes a dramatic increase in incident hypertension, suggesting a protective effect of estrogen on blood pressure. In female rats, estrogen has been shown to decrease sympathetic nerve discharge (SND) and Blood pressure. However, SND was not recorded during estrogen replacement therapy in human. In normotensive postmenopausal women, chronic transdermal use of estrogen replacement therapy decreased SND without augmenting arterial baroreflexes and caused a small but statistically significant decrease in ambulatory BP. Sympathetic inhibition was evident only with chronic rather than acute estrogen administration. This may be an important consideration in optimizing the beneficial effects of hormone replacement therapy on blood pressure and overall.

Valenta *et al.* (2001) developed and evaluated the possible use of polycarbophil-Cysteine (PCP-Cys) as polymer matrix for transdermal progesterone application. Thiolated polycarbophil was synthesized by the covalent attachment of cysteine to the basic polymer. The adhesive properties of PCP-Cys in comparison to polyvinylpyrrolidone/ hydroxy propylmethylcellulose (PVP/HPMC) and polyvinylpyrrolidone/ polyvinyl alcohol (PVP/PVA) were investigated by testing of total work adhesion on porcine skin. The film based on PCP-Cys displayed very high cohesive properties due to the formation of interchain disulphide bond. PCP-Cys, a partially

thiolated polymer, might be a novel polymer matrix for transdermal progesterone delivery with excellent adhesiveness on porcine skin.

Akkad *et al.* (2001) examined the effect of continued transdermal estrogen replacement therapy on serum level of secreted vascular endothelial growth factor in women following hysterectomy and Oophorectomy. They found that transdermal estrogen replacement in hysterectomized and oophorectomyized women appears to be associated with a significant reduction in serum vascular endothelial growth factor (role in preservation and restoration of endothelial integrity).

Gussinye *et al.* (2001) studied the effect of long-term estradiol therapy on oral bone mineral density values in Turner syndrome patients and observed beneficial effect in such patients.

Slatter *et al.* (2001) compared the serum estrone sulphate level in postmenopausal women during long term treatment with commonly prescribed doses of oral and transdermal estradiol. They found that there was only small increase in estrone sulphate levels after transdermal estradiol therapy as compared to oral estradiol levels because of low bioavailability of oral estradiol compared with transdermal doses.

Audet *et al.* (2001) evaluated the contraceptive efficacy and cycle control of transdermal contraceptive patch and oral contraceptives. They observed that the contraceptive patch was comparable to a combination oral contraceptive in contraceptive efficacy and cycle control. The patient compliance was better with the weekly contraceptive patch than oral contraceptive.

Vahkavavarra *et al.* (2001) studied the effect of oral and transdermal estradiol replacement therapy on markers of coagulation, fibrinolysis, inflammation and serum lipids and lipoproteins in postmenopausal women. The oral estradiol increased the level of fibrinolytic activity, decreased serum soluble E-selectin levels and induced potentially antiatherogenic changes in lipid and lipoproteins. In contrast to these beneficial effects, oral estradiol changed the markers of coagulation towards hypercoagulabilty and increased serum C reactive protein. Transdermal or placebo had no effects on any of these parameters.

Godsland *et al.* (2001) estimated the effects of different hormone replacement therapy regimens on lipid and lipoproteins levels. They found that all estrogens given alone raised HDL cholesterol and lowered LDL and total cholesterol. Oral estrogens raised triglycerides. Transdermal estradiol 17-beta lowered triglycerides. Progesterone

had little effect on estrogen-induced reductions in LDL and total cholesterol. Estrogen induced increases in HDL and triglycerides were opposed according to the type of progestogen. Route of estrogen administration and type of progesterone had shown differential effects of hormone replacement therapy on lipid and lipoprotein levels.

Baracat *et al.* (2001) examined the ultra-structure features of endometrium cells the postmenopausal women of treated with different doses of conjugated equine estrogen or transdermal 17-beta estradiol. They found that the ultra-structure proliferative changes of the postmenopausal endometrium induced by 1.25 mg/day of conjugated equine estrogen were similar to 50 microg/day of transdermal 17-beta estradiol.

West *et al.* (2001) compared the transdermal versus oral estrogens on vascular resistance index, mean arterial pressure, serum lipid concentrations, norepinephrine, and left ventricular structure. They reported an equivalent reduction in these parameters by both the treatments.

Nicoli *et al.* (2001) designed, triptorelin loaded nanospheres for transdermal iontophoretic administration, which on the basis of particle size and drug entrapment were shown to be suitable for transdermal iontophoretic administration.

Kulkarni *et al.* (2001) studied the effects of estrogen to modulate both dopamine and serotonin neurotransmitter systems (pathogenesis of schizophrenia). They found that 100-microg adjunctive transdermal estrogen significantly enhanced the treatment of acute, severe psychotic symptoms in women with schizophrenia. The differential response of adding 50 mcg verses 100 mcg estradiol on the type of symptoms affected may be related to the estrogen effect on LH and prolactin. The positive impact of estrogen treatment of psychotic symptoms by a direct effect on dopamine and serotonin systems or via indirect prolactin mediated effect may be very useful in the overall treatment with schizophrenia.

Fang *et al.* (2001) studied the in-vitro skin permeation of estradiol from various proniosome formulations. Proniosomes with span 40 and span 60 increased the permeation of estradiol across skin. Both penetration enhancer effect of non-ionic surfactant and vesicle skin interaction were contributed to mechanism of proniosomes to enhance estradiol permeation. The types and content of nonionic surfactant in proniosomes were found to be important factors affecting the efficacy of transdermal estradiol delivery.

Anbacher *et al.* (2001) concluded the pharmacokinetics and efficiency of different estrogens administered orally or applied transdermally. The estrogens, which elicit a marked hepatic response, induced greater beneficial effects on serum lipoproteins than transdermal estrogens, which circumvent first pass liver metabolism. Oral conjugated estrogens and transdermal estradiol increased the bone density and prevented the bone loss.

Koch *et al.* (2001) studied the allergic contact dermatitis from estradiol and norethisterone acetate in transdermal hormonal patch and observed no untoward effects.

Karjalainen *et al.* (2001) studied the effect of peroral and transdermal estrogen replacement therapy on glucose and insulin metabolism and observed that neither peroral nor transdermal oestradiol replacement therapy seemed to induce any negative effects on glucose metabolism over a time period of six months.

Falco *et al.* (2001) studied the fibrinolysis and changes in lipoprotein levels in women with coronary artery disease. Epidemiological studies have shown decreased risk of cardiovascular disease in postmenopausal women on treatment with hormone replacement therapy, which could be attributed to modification in the fibrinolytic system and lipoproetin levels. An increase in fibrinolystic activity and decrease in PAI-I and Lp levels were observed in CAD women receiving transdermal hormone replacement therapy and these changes might had a have favorable impact on the risk of new cardiovascular disease.

Kos-kudla *et al.* (2000) studied the effect of hormone replacement therapy on postmenopausal asthmatic women and found a greater reduction in estrogen in asthmatic women than in postmenopausal healthy women. They observed diminishing psychosomatic symptoms of the menopause and symptoms of asthma.

Czarnecka *et al.* (2000) studied the influence of hormone replacement therapy on the quality of life in postmenopausal women with hypertension and observed that three month hormone replacement therapy in hypertensive postmenopausal women slightly improved the general well being, seemed to decrease emotional tension, increased sexual capacity and markedly relieved some vasomotor symptoms.

Vahkavaara *et al.* (2000) attended to explain weather the vascular effects of estradiol depend on the route of administration by comparing the effects of oral estradiol and transdermal placebo, transdermal estradiol and oral placebo on in-vivo endothelial function. They found that oral but not transdermal estradiol induced potentially

antiatherogenic changes in in-vivo endothelial dependant vasodilatation and lipid concentration.

Seeger *et al.* (2000) studied the effect of norethisterone acetate on estrogen metabolism in postmenopausal women. Dominance of estradiol metabolism at D-ring over the A-ring metabolism may play a role in the pathophysiology of human breast carcinogenesis. They suggested that transdermal norethisterone acetate in hormone replacement therapy did not elicit any change in estrogen metabolism after 2 weeks treatment. However, oral norethisterone may in some cases have an impact on estradiol metabolism, which should be further evaluated.

Doren *et al.* (2000) updated the clinical trials since 1995 on prevention of postmenopausal osteoporosis with estrogen replacement therapy associated compounds.

Cagnacci *et al.* (2000) perform the different circulatory response to melatonin in postmenopausal women without and with HRT. In young men and women, melatonin influences vascular reactivity and reduces blood pressure and morepinephrine levels. They suggest that the circulatory response to melatonin is conserved in postmenopausal women on HRT but not in untreated postmenopausal women.

Taggart *et al.* (2000) studied the effect of site of application on the transcutaneous absorption of 17-beta estradiol from a transdermal delivery system (Climara). They found that the extent of absorption was significantly more from buttocks than from abdomen application. The application to buttocks may provide an advantage in women who experience menopausal symptoms at the end of week.

De leo *et al.* (2000) assessed the effects of bilateral Oophorectomy on the resting ECG and found that Oophorectomy induced significant though not clinically evident modifications in resting ECG. These ECG changes may be probably due to the sudden reduction in sex hormone plasma levels after ovariectomy. Administration of estradiol induced regression of the ECG modifications.

Peebles *et al.* (2000) evaluated the effects of hormone replacement therapy on seizure activity in postmenopausal women with epilepsy. A weekly transdermal patch of estradiol 0.1 mg/d was initiated for the first three months. During second-third months, the regimen was expanded to include oral dose of medroxyprogesterone acetate 2.5 mg once daily. Antiepileptic medications and their dosage remained constant. They found that hormone replacement therapy decreased the incidence of seizures, cessation of vasomotor flushing, improved sleep and positive impact on the lipid profile.

McKeever *et al.* (2000) evaluated the efficiency, safety, and tolerability of 2 years application of an estradiol matrix transdermal system for prevention of bone loss in postmenopausal women. They found that estradiol matrix transdermal system was effective in preventing postmenopausal bone loss at dosage of 0.025 to .01 mg/d and had a safety profile consistent with the known effects of estrogen/ progestins.

Colacurci *et al.* (2000) studied the effects of hormone replacement therapy on postmenopausal uterine myoma. They found that the sequential continuous HRT did not increase the volume of uterine myoma.

Catz *et al.* (1990) studied the in-vitro evaluations of transdermal levonorgestrel. They found that the devices deliver levonorgestrel and the enhancer at about the same rate regardless of the skin type. It appears that the flux of LN follows the ethyl acetate until the devices are nearly depleted of ethyl acetate, when delivery of LN remains relatively high.

Fauth *et al.* (2002) studied the adhesive backing foil interaction affecting the elasticity, adhesion strength of laminates, and how to interpret these properties of branded transdermal patches. Seven branded transdermal patches (Alora, Cutanum, Estraderm MX 50, Estraderm TTS 50, Fem-50 Monorest, Oesclim) were included in the investigation. They observed that the branded patche no correlation was found between young's modulus and both the peel force on stainless steel and the skin adhesion reported in the studies.

Rafiee-Tehrani *et al.* (2001) developed a transdermal patch using acrylic resin (Eudragits E 100, RSPO, and RLPO) as adhesive and rate controlled polymers. The effect of two plasticizers, acetyltriburyl citrate and triethyl citrate on the release patterns of 17-beta estradiol form TDS formulation and they were almost identical. The effect of two different release modifiers, propylene glycol and myristic acid on the release pattern of 17-beta estradiol from prepared unilaminated devices was evaluated. It was shown that the use of these release modifiers significantly increased the release of 17-beta estradiol from a transdermal unilaminated patch. They suggested that the acrylic polymers are suitable polymer for the preparation of 17-beta estradiol TDS adhesive devices.

Valenta *et al.* (2001) studied the effect of urea and pentothenol on the permeation of progesterone through excised rat skin from polymer matrix. They found that the highest permeation rate were measured from polyvinyl alcohol matrices containing 5% urea and from polyvinylpyrrolidone (PVP) containing 6% Pentothenol.

Kos-Kudla *et al.* (2001) performed circadian serum levels of dehydroepiandrosterone in postmenopausal asthmatic women before and after long-term hormone replacement therapy. They suggested that postmenopausal asthmatic women show diminished circadian dehydroepiandrosterone sulphate serum concentrations irrespective whether they were treated with glucocorticoids or not. However, after 6 months of hormonal replacement therapy in these groups increased levels of dehydroepiandrosterone were found.

Muller *et al.* (1996) studied the bioavailability of estradiol from a new matrix and a conventional reservoir type transdermal therapeutic system. They found that MTTS proved to be equivalent to the RTTS with respect to the extent of absorption. Due to the differences in patch design and composition, the rate of absorption was different between the two systems with less fluctuating E2 plasma level during application of the matrix system. Local tolerability and adhesion of MTTS appeared to be better than those of the reservoir system.

Salbach *et al.* (2000) investigated the influence of hormone replacement therapy on body mass index (BMT) and serum leptin level. They suggested that the low dose, transdermal HRT exhibited no influence on serum leptin levels or BMI in postmenopausal women. These findings suggested that the low dose HRT did not influenced body weight regulation in postmenopausal women.

Gokmen *et al.* (1999) evaluated the link between HRT and lipid lipoprotein concentration. They found that the cumulative evaluation of on estrogen only replacement therapy revealed a significant decrease in T.Ch, LDL-C and triglycerides and increase in HDL-C, however the increase in HDL-C, and triglycerides was significantly higher in conjugated equine estrogen than TDE2. Cumulative evaluation of on estrogen and progestins hormone replacement therapy revealed a significant decrease in T.Ch, LDL-C and increase in HDL-C for all; however, triglycerides and VLDL levels decreased in TDE2 + MPA and TDE2+DD (dydrogestrone) groups.

Basbug *et al.* (1997) compared the effect of continuous noncombined transdermal estradiol versus oral conjugated estrogen on serum sex hormone- binding globulin (SHBG) levels prior to and during the 10^{th} and 22^{nd} weeks of therapy in patients with surgical menopause. They found that transdermal estrogen ha no effects on SHBG, whereas oral conjugated estrogens cause considerable increase.

Vavilis *et al.* (1997) studied the effect of transdermal estradiol on conjunctiva in postmenopausal women. They suggest that a significant increase of estradiol levels and of vaginal maturation changes in conjuctival epithelium were also observed. These data supports the view that the hormonal replacement therapy induces cytological maturation changes in conjuctival epithelium in postmenopausal women.

Pornel *et al.* (1996) studied the efficacy and safety of Monorest in two positive controlled clinical trials. They found that Monorest appears to be as equally effective as oral and transdermal oestradiol as far as reduction in the incidence and severity of menopausal symptoms was concerned. It was safe and also better tolerated than Estraderm.

Marty (1996) developed transdermal system, Monorest is one of new generation of patches, in which estradiol was dispersed in a micronised suspension throughout the adhesive matrix. This design results a very thin patch with good cosmetic acceptability. They found that the standard reservoir patch, Estraderm, Monorest maintained plasma estradiol concentration at or above the target level of 40pg/ml throughout the 84-h dosing interval.

Tufekci *et al.* (1993) studied the effect of progestogens on estrogen-induced lipoprotein change and they found that medroxyprogesterone acetate, whether used continuously or sequentially, dose not oppose the beneficial effects of transdermal 17-beta estradiol on the lipoprotein profile. 344

Wemme *et al.* (1995) studied the effect of oestrogen/gestagens replacement therapy on liver enzyme in patients with Ullrich-Turner syndrome. The absence of breast development and prevention osteoporosis in Ullrich-Turner syndrome requires estrogen/gestagens substitution therapy. They suggested that the patients with Ullrich-Turner syndrome on oral oestrogen replacement therapy are more susceptible to develop increased serum liver enzyme levels as compared with eukaryotic females treated with the same estrogen preparation for other disorders.

Lipp *et al.* (1999) studied the use of x-ray crystallography for characterization of single crystals grown in steroid containing transdermal drug delivery system. They suggested that steroid containing matrix transdermal drug delivery system should be stabilized against drug re-crystallization e.g. by addition of suitable crystallization inhibitors, furthermore, systems containing estradiol may be stabilized be efficient removal water.

Colacurci *et al.* (2001) studied the effect of a short-term suspension of hormone replacement therapy on mammographic density. They found that suspension of hormone replacement therapy for about 3 weeks may reverse mammographic density increase associated with its use.

Godsland *et al.* (2001) reviewed the effects of postmenopausal hormonal replacement therapy on lipid, lipoprotein, and apolipoprotein concentrations: analysis of studies published from 1974 to 2000. They suggested that all estrogens alone regimens raised HDL cholesterol and lowered LDL and total cholesterol. Oral estrogens raised triglycerides. Transdermal estradiol 17-beta lowered triglycerides. Progestogens had little effect on estrogen-induced reduction in LDL and total cholesterol. Estrogen induced increased in HDL and triglycerides were opposed according to the type of progestogens, in the order of least to greatest effect: dydrogesterone and medrogestone, progesterone, cyproterone acetate, medroxyprogesterone acetate, transdermal norethindrone acetate, norgestrel, and oral norethindrone acetate. Tibolone decreased HDL cholesterol and triglycerides levels. Raloxifene reduced LDL cholesterol levels. HRT generally lowered lipoprotein. Finally the route of estrogen administration and type of progestogen determined differential effects of HRT on lipid and lipoprotein levels.

Agrawal *et al.* (2000) studied the effect of hormone replacement therapy on serum vascular endothelial growth factor concentrations in postmenopausal women. They found that postmenopausal women with uterus in-situ and those who received hormone replacement therapy had higher vascular endothelial growth factor concentrations than did those who had hysterectomies and who did not receive hormone replacement therapy. Among women receiving hormone replacement therapy, those who receiving conjugated equine estrogens alone had higher VEGF concentration. This estrogen-mediated increase in serum VEGF concentration may be the mechanism by which hormone replacement therapy benefits the cardiovascular system.

Mikkola *et al.* (2000) administered transdermal estrogen without progestins increases the capacity of plasma and serum to stimulate prostacyclin production in human vascular endothelial cells. They found that transdermal hormone replacement therapy during E2 only phase increased the capacity of plasma and human serum production of vasoprotective prostacyclin in human vascular endothelial cells, without affecting production of endothelin-I. Addition of norethisterone acetate prevented the stimulation.

Exacoustos *et al.* (2000) assessed uterine arterial blood flow and endometrial thickness in postmenopausal patients receiving sequential hormone replacement therapy at different phase of treatment. They concluded that the decrease in endometrial thickness in phase 0 suggests protective effects of cyclic sequential regimen on the endometrium. Dydrogesterone did not interfere markedly with vasodilatory effect of estrogen on uterine arteries.

Ylikorkala *et al.* (1998) studied the long-term effect of oral and transdermal postmenopausal hormone replacement therapy on nitric oxide, endothelin-1, prostacyclin, and thromboxane. They compared the long-term effect of sequential oral hormone replacement therapy and transdermal hormone replacement therapy on vasodilatory nitric oxide and prostacyclins as well as vasoconstrictive endothelin and thromboxane A2. All of which may be the factors in the protective effect of hormone replacement therapy against cardiovascular disorders. Both regimen increased plasma estradiol level and alleviated vasomotor symptoms. Neither regimen caused significant change in nitrate, endothelin-1, prostacyclin, or thromboxane A2 in nonsmoking women. Female smokers had significantly higher levels of endothelin-1, which were significantly reduced by transdermal HRT at 6 months of treatment.

Cicinelli (1998) assessed the effect of short-term transdermal E2 administration on nitric oxide (NO) plasma level in postmenopausal women. They suggested that during 1 week treatment regimen with transdermal E2, plasma level of NO in postmenopausal women were significantly higher than baseline level on days 2, 3, and 6. They reported that the effect of estrogens on NO synthesis is rapid and that it is maintained with repeated administration.

Sener *et al.* (1996) evaluated and compared the effect of two different modalities of hormone replacement therapy on the size of uterine fibroids. They found that hormone replacement therapy with 50 μg transdermal E2 plus 5 mg MPA increased the size of the uterine fibroids.

Krasnow *et al.* (1996) compared the effect of oral micronised E2 with transdermal E2 on endometrial receptivity in women undergoing oocyte donation. They found that supraphysiologic serum E2 level associated with oral micronised E2 might have a deleterious impact on endometrial receptivity. The development of more physiologic hormone replacement protocols may enhance endometrial receptivity and lead to improved clinical pregnancy rates.

Ginsburg (1995) studied the effect of ethanol on the clearance of estradiol in postmenopausal women. They suggested that the ethanol ingestion might decrease E2 clearance after removal of transdermal E2 patches.

Suhonen (1992) performed the endometrial effect of transdermal estradiol and progestins ST-1435 in postmenopausal women and they suggested that when estrogen stimulation was adequate, the transdermal ST-1435 induced a progestins effect on endometrium, i.e. it had an end organ effect.390

Smith (1992) evaluated the effect of vehicle supplementation on serum estradiol pharmacokinetics of Estraderm patch. They found that the addition of ethanol to the Estraderm patch increased the duration of elevated serum E2 level measured in menopausal women, thus potentially increased the effective life span of the transdermal therapeutic system.

D'Amico *et al.* (1991) studied the induction of hypothalamic opioid activity with transdermal estradiol administration in postmenopausal women. They suggested that E2, in physiologic concentration, induced hypothalamic opioid activity.

Hung *et al.* (1989) studied the feasibility and efficacy of natural 17 beta-estradiol and progesterone to induce endometrial changes. They suggested that transdermal E2 patches and vaginal suppositories, while being as effective in inducing endometrial development, for the embryo transfer procedure with donor ovum as synthetic steroids, can also provide a more physiologic approach that may conveniently and safely be extended into the second trimester of pregnancy.

Friend *et al.* (1991) studied the coetaneous effect of transdermal levonorgestrel and penetration enhancers i.e. ethyl acetate with or without ethanol. They found that the histological evaluation of the application site of two of the formulation confirmed the visual observations of mild sub acute irritation. The changes produced by transdermal levonorgestrel were reversible. The problem of skin irritation of transdermal devices is discussed with particular reference to the use of ethyl acetate and ethanol as skin penetration enhancers.

Kos-Kudla *et al.* (2001) studied the effects of hormone replacement therapy on endocrine and spirometric parameter in asthmatic postmenopausal women. They concluded that hormone replacement therapy in postmenopausal asthmatic women has a favorable influence on the course of asthma, reduces daily use of glucocorticocoids and

frequency of asthma exacerbations and normalizes serum concentration of estradiol, cortisol, and dehydroepiandrosterone, which were decreased before HRT.

Piccinini *et al.* (2000) studied the indirect evidence that estrogen replacement therapy stimulates nitric oxide synthase in postmenopausal women. They suggested that transdermal estradiol replacement therapy in postmenopausal women was able to stimulate NO production through the involvement of endogenous l-arginine. They showed linear correlation between BMI and the citrulline / arginine ratio, and additional protective cardiovascular effect in overweigh women.

Battaglia *et al.* (1999) evaluated the plasma thromboxane and plasma viscosity in relation with color Doppler flow parameter in postmenopausal women treated with hormone replacement therapy. They found that plasma thromboxane levels and plasma viscosity were significantly reduced. Significant correlations were found between thromboxane plasma concentrations, plasma viscosity, and uterine artery resistance. Thus hormone replacement therapy seems to be responsible for both direct and indirect modifications at the level of the vessel wall physiology.

Haenggi *et al.* (1999) reported the decreased level of serum endothelin with postmenopausal hormone replacement therapy or tibolone. Endothelin was the potent vasoconstrictor peptide known to date. Hormone replacement therapy with estrogen reduces plasma endothelin levels. They found that tibolone decreased endothelin level to a similar degree as conventional estrogen-progestogen-replacement therapy. These data provide another potential mechanism supporting the cardio-protective effects of tibolone.

Altiery *et al.* (1997) studied the bone density changes in postmenopausal women with administration of ipriflavone alone or in association with low-dose estrogen replacement therapy. Ipriflavone is a synthetic flavonoid that has been shown to exert a direct inhibitory effect on osteoclastic activity and possibly stimulate the osteoblast activity in different experimental models. They found that lower transdermal estrogen dose is unable to increase the anti-resorptive effect of ipriflavone and did not exert any further action in the prevention of postmenopausal osteopenia.

Massafra *et al.* (1996) studied the changes in the erythrocyte antioxidant enzyme system during transdermal estradiol therapy for secondary amenorrhea. They suggested that estrogens restore to physiological plasma level, stimulate erythrocyte antioxidant GSH-Px activity, improving the antioxidant power of amenorrheic women.

Tranquilli *et al.* (1995) evaluated the effect of the different components of hormone replacement therapy on the production of free radicals in platelets membranes from menopausal women. They concluded that reduction of lipid peroxidation during hormone replacement therapy was not only due to estrogens, but also depends upon the combined action of sex steroids. This observation justified not only the combined regimen (estrogen plus progestin) in hormone replacement therapy, but also the positive effect of progestins alone on patients who cannot use estrogens.

Shimoya *et al.* (1999) examined the effect of transdermal estrogen therapy on the endometrium thickness and serum hormone level in anovulatory patients treated with clomiphene citrate. They found that addition of transdermal estradiol to the treatment protocol of the women treated with clomiphene citrate elicited a favorable response of the endometrium.

Ginsburg *et al.* (1998) reported the half-life and metabolism of estradiol in postmenopausal women. They observed that the apparent half-life of estradiol after discontinuing a transdermal estradiol patch was 2.7 h or 161 min.

Brunelli *et al.* (1996) studied the effect of hormone replacement therapy on various immune cell subset and natural cytotoxicity. They found that natural killer cell function was transiently increased on 8 day and greatly reduced on day 21 during the first month of therapy.

Modena *et al.* (1999) studied the effect of transdermal 17-estradiol on left ventricle anatomy and performance in hypertensive women. They found that transdermal 17-beta estradiol which is associated with antihypertensive therapy, may contribute in the reduction of left ventricular mass in hypertensive postmenopausal women.

Pinto *et al.* (1997) evaluated the effect of endogenous estrogen and acetylcholine-induced vasodilation in fertile normotensive women. They suggested that exogenous estrogens restore acetylcholine –induced vasodilation.

Corson *et al.* (1993) reported clinical experience with Systen, a new transdermal form of hormone replacement therapy. They compared the release rate of Systen with Estraderm. Both patch were effective and well tolerated. However, Systen was associated with fewer adverse skin reactions and was significantly superior to Estraderm with respect to patch adhesiveness and patients assessment of treatment convenience. These differences between the two patches reflect progressive refinement in TTS technology.

Koh *et al.* (1997) investigated the effect of estrogen with antioxidant potential on soluble markers of chronic vascular inflammation by administering the 17-beta estradiol with or without the medroxyprogesterone acetate. They suggested that hormone replacement therapy significantly lowered intracellular adhesion of molecules-1 level by 8% and tended to lower E-selectin levels.

Hirvonen, *et al.* (1997) studied the effect of transdermal estrogen therapy in postmenopausal women: a comparative study of an estradiol gel and a transdermal delivery patch. They suggested that both the estradiol gel and the transdermal delivery patch are equally effective in hormone replacement therapy but the gel preparation was less irritative to the skin.

Bachhi-Modena *et al.* (1997) studied the efficacy and tolerability of Estraderm MX a new estradiol matrix patch. They found that Estraderm MX 50, a new matrix patch, offered an effective and well-tolerated dosage form for transdermal delivery of 0.05 mg estradiol per day.

Deo *et al.* (1997) developed mesophasic proliposomal system for levonorgestrel and evaluated both in-vitro and in-vivo. They found that the release rate was higher with alcohol and then with lemon oil. This system was however, superior to the PEG-based ointment system, which was employed as the control formulation. The results demonstrate the potential of proliposomal system for efficacious transdermal delivery of hydrophobic drugs.

Hanggi *et al.* (1997) compared trans-vaginal ultra-sonography with histological findings in endometrial evaluation of postmenopausal women using hormone replacement therapy and also evaluated endometrial safety of three hormone replacement therapy regimens. They found that the trans-vaginal ultra-sonography of the endometrium reliably predicts the histological picture in the hormone replacement therapy users. All the three-hormone replacement therapy was safe with respect to endometrium.

Basbug *et al.* (1997) compared the effect of continuous noncombined transdermal estradiol versus conjugated estrogen on serum sex hormone-binding globulin level prior to and during the 10^{th} and 22^{nd} weeks of the therapy. They reported that transdermal estrogen had no effect on serum sex hormone-binding globulin level, where as oral conjugated estrogens causes considerable increase.

Connell (1997) reported transdermal estrogen therapy. Estrogen hormone replacement therapy is becoming an important weapon in the fight against osteoporosis

and heart disease in postmenopausal women, in addition to its original; role of alleviating many of the symptoms associated with menopause. Dr. Connell discussed various benefits of estrogen hormone replacement therapy and advantages of transdermal administration of estrogen appear to offer over other routes of administration.

Wollter-Svensson *et al.* (1997) investigated the clinical effects especially with regards to hitopathological and bleeding patterns by levonorgestrel released from an intrauterine device in combination of estradiol valerate or transdermal estradiol in perimenopausal women. They found that continuous combined hormone replacement therapy was well accepted in perimenopausal women when the progesterone was given an IUS.

Parra *et al.* (1997) assessed the effect of estrogen replacement on the stimulation blockade of the dopaminergic (DA) and opiodergic neural control of hypothalamic-gonadotropic function in postmenopausal women. They found that in the untreated postmenopausal women, the dopaminergic system has little and the opiodergic system has no significant input in the control of gonadotropins or prolactin release. However, following estrogen replacement, opoid are involved in the inhibition of LH release and stimulating prolactin release, while the dopaminergic system acts to inhibit prolactin release and modulates LH release or inhibition, depending on the level of circulating estrogens.

De Leo *et al.* (1997) studied the inhibition of ovulation with transdermal estradiol and oral progestogens in perimenopausal women. They found that combined hormone replacement therapy with low dose of transdermal estrogen patch and different oral progestogens reduced the menopausal symptoms and also safeguards against unwanted pregnancies in the perimenopausal women.

Erianne *et al.* (1997) compared the local tolerability and adhesion of two transdermal systems for estradiol delivery using membrane system (Estraderm -50) and a new matrix system (Monorest). They suggested that there was no difference between the local tolerability and adhesion of an established membrane system for estradiol delivery (Estraderm -50) and a newly introduced matrix system (Monorest).

Vavilis *et al.* (1997) evaluated the effect of hormone replacement therapy on the conjunctiva in postmenopausal women. They reported that the hormonal replacement therapy induces cytological maturation changes in conjunctival epithelium in postmenopausal women.

Pinto *et al.* (1997) studied the effect of estrogen replacement therapy on thrombin generation. They found that unopposed estradiol given by transdermal route induced a slight but significant blood clotting activation, which seems strictly related to its biological activity.

Schunkert *et al.* (1997) studied the effect of estrogen hormone replacement therapy on the rennin-angiotensin system in postmenopausal women. The effect of such therapy on rennin, ACE, and aldosterone was less clear. They reported that only rennin suppression was seen with either oral or transdermal estrogen replacement therapy finally, only marginal differences between groups were observed for serum ACE activity and aldosterone.

Rozenbaum *et al.* (1996) compared the local skin tolerability of a matrix type estradiol transdermal system, Oesclim 50, with that of the reservoir-type system, Estraderm. They fond in the first study, neither Oesclim 50 nor Estraderm 50 induced allergic reactions. In the second study, the local tolerability of Oesclim was significantly better that of Estraderm, in terms of the number, duration and severity of the application site reaction.

Foidart *et al.* (1996) studied the use of transdermal estradiol (Estraderm TTS) in hypertensive postmenopausal women.

Masssafra *et al.* (1996) studied the changes in the erythrocyte antioxidant enzyme system during transdermal estradiol therapy for secondary amenorrhea. They suggested that estrogen restored to physiologic plasma level, stimulate erythrocyte antioxidant GSH-Px activity, improving the antioxidant power of amenorrheic patients.

Mitchell *et al.* (1996) discussed the replacement therapy for arginine vasopressin (AVP), growth hormone (GH), cortisol, thyroxin, testosterone and estrogen.

Habiba *et al.* (1996) assessed the changes in lipoprotein, apoproteins and coagulation factor, induced in post-menopausal women treated by a new transdermal oestradiol patch. They found that the transdermal route of estradiol administration cause minimal changes in lipoprotein metabolism. The statistically changes in the thrombophilia profile parallel those observed with oral hormone replacement therapy but, similarly, may not reflect clinical significance.

Vermeire *et al.* (1996) studied the sucrose laurate gels as a percutaneous delivery system for estradiol in rabbit. They found that significant increase in skinfold thickness was seen for the skin biopsies treated with gel containing 15% sucrose laurate. It can be

concluded that sucrose laurate showed a potential as an absorption enhancer for percutaneous drug delivery.

Lippert *et al.* (1996) studied the serotonin metabolic excretion after postmenopausal estradiol therapy. Serotonin, known for its beneficial action on mood and well-being was also involved in cardiovascular functions. They found that the enhancement of serotonin turnover may contribute not only to an improvement of mood and well being but also to a cardio-protective effect of estradiol observed after hormone substitution in postmenopausal women.

Albertsson (1996) investigated the acute effect of transdermal estradiol-17 beta on exercise-induced angina and ST augment depression in syndrom-X. They concluded that transdermal estradiol 17- beta has a beneficial effect on myocardial ischemia in postmenopausal women with syndrom-X and may be a useful therapeutic agent in this disease.

Gregoire *et al.* (1996) studied the transdermal oestrogen for treatment of severe postnatal depression. They found that transdermal estrogen is an effective treatment for the postnatal depression.

Matuszewski *et al.* (1996) investigated the cholesterol and its fractions in women after ovariectomy treated with Estraderm patch. They found that after treatment the mean value of HDL was increased, and decreased in mean value of triglycerides. Changes in mean level of LDL and total cholesterol were not statistically significant.

Lein *et al.* (1996) investigated the hormone therapy and phytoestrogens. They reported alternative therapies for menopausal symptoms with Chinese traditional herbs, and a comparison of the molecular structures of phytoestrogens with estradiol and diethylstilbestrol was examined. The list of herbs and foods reported to elicit an oestrogenic response in animals was compiled.

Castelo-Branco *et al.* (1996) studied the effect of hormone replacement therapy on bone mass in patients with ovarian failure due to bone marrow transplantation. They suggested that hormone replacement therapy was safe for these patients and that such therapy should initiated after transplantation in women to prevent adverse effects of long-term ovarian failure.

Oelkers *et al.* (1996) studied the effect of estrogens and progestogens on the rennin-aldosterone system and blood pressure. They found that combined use of estradiol with synthetic progestogens might therefore enhance estrogen effect on body sodium and

blood pressure. A new progestogen (Drospirenone) with an antimineralocarticoids effect like that of progesterone was described that slightly lowered body weight and blood pressure in a contraceptive formulation together with estradiol.

Marty *et al.* (1996) investigated the Monorest transdermal patch: technical development and pharmacokinetic profile. They found that after the pharmacokinetics studies, that Monorest allowed transdermal release of estradiol at a constant and reproducible rate at doses from 25-100 μg/day. In contrast to the standard reference reservoir patch, Estraderm, Monorest maintained plasma estradiol concentration at or above the target level of 40pg/ml throughout the 84-h dosing interval.

Setniker *et al.* (1996) compared the bioavailability of estradiol from two transdermal patches. The bioavailability of estradiol from a new matrix type estradiol transdermal patch (Dermestril: Test Patch) was compared to that of the widely used liquid reservoir membrane controlled type transdermal patch. They concluded that the test patch can be considered practically bioequivalent to the reference patch with regard to the extent of absorption, but not with regard to the rate of absorption, because the estradiol concentration in serum are more constant during the application of the test transdermal patch than during the application of the reference.

Tikkanen *et al.* (1996) reviewed the recent literature concerning the effect of menopause and hormone replacement therapy on blood plasma lipoprotein and hemostatic system, as well as on the interaction between these two coronary heart disease risk factor systems. They concluded that numerous existing hormone replacement therapy regimens may provide alternatives and useful possibilities, further studies are needed concerning (a) novel progestins with minimal HDL cholesterol lowering effects, (b) transdermal and other non-oral routes for hormone replacement therapy, (c) possible antioxidative properties of estrogen and (d) metabolic links between the lipoprotein and hemostatic risk factor system.

Marty *et al.* (1996) studied the new trends in transdermal technologies by developing the skin patch Monorest. They found that the standard reference reservoir patch, Estraderm, or another matrix patch, Systen/Evorel, Monorest maintained plasma estradiol concentrations at or above target level of 40 pg/ml throughout the 80-h dosing interval.

Muller *et al.* (1996) compared the bioavailability of estradiol from a new matrix patch with that of the conventional reservoir system. They found that the matrix type

transdermal system proved to be equivalent to the reservoir type transdermal system with respect to the extent of estradiol absorption. Due to difference in patch design and composition, the rate of absorption was different between the two systems, with less fluctuating estradiol plasma levels during application of the matrix system. Local tolerability and adhesion of MTTS appeared to be better than those of the reservoir system.

Lippert *et al.* (1996) studied the influence of estradiol treatment on urinary excretion of relaxin. They found that the transdermal but not with oral administration, a significant increase of urinary relaxin excretion was registered.

El Sayed *et al.* (1996) studied the systemic sensitization of 17-beata estradiol induced by transcutaneous administration. They concluded that contact eczema due to transdermal therapeutic systems was usually caused with agents other than the active drug raised the risk of generalized eczema and subsequent systemic complications. This risk must be taken into account when prescribing substitution hormone replacement therapy for menopause.

Aune *et al.* (1995) investigated the effect of hormone replacement therapy on the reactivity of monocytes and platelets in whole blood, measured by tissue factor activity, tumor necrosis factor-alpha and thromboxane B2. They found that the twelve months of hormone replacement therapy reduced cellular activation of blood monocytes and platelets; these changes may accounted for some of the beneficial effects in reducing the risk of cardiovascular disease.

Irion *et al.* (1995) measured the effect of a combination of excipients (propylene glycol monolaurate, PGML) from a silicone based pressure sensitive adhesive on drug transport across the skin. They found that the PGML and estradiol transport showed a good correlation over 3 days, suggesting that the two species was co-transported across the epidermis.

Araya *et al.* (1995) studied the effects of a 17-beta estradiol gel preparation on hormone level in postmenopausal women. They observed a significant increase in estradiol level and decrease in FSH level. Estrone level also increased but the estradiol/estrone ratio was maintained in values over 1. No changes in SHBG or IGF-1blevels were observed. They concluded that the gel increased serum estradiol level over 60pg/ml in four of six women that there was big individual variability and the application zone could influence the serum estradiol level achieved.

Pierard Franchimont *et al.* (1995) studied the skin water-holding capacity and transdermal estrogen therapy for menopause. The aim of this study was to model and measure the influence of estrogen transdermal hormone replacement therapy on subtle physiological changes taking place in the epidermis during the perimenopause. They found that the water holding capacity of the stratum corneum was significantly increased at the plastic occlusion stress test site in women receiving transdermal estrogen.

Sloand *et al.* (1995) studied the beneficial effect of low dose transdermal estrogen on bleeding time and clinical bleeding in uremia. They concluded that transdermal application of 17-beta estradiol was safe and effective by means to reduce bleeding time and clinical hemorrhage in patients with renal failure and prolonged bleeding time.

Ginsburg *et al.* (1995) studied the effect of ethanol on the clearance of estradiol in postmenopausal women. They found that ethanol ingestion decreased estradiol clearance after removal of transdermal estradiol patches.

Lindgren *et al.* (1995) investigated the transdermal hormonal replacement therapy with transdermal progestins every second month. They concluded that the transdermal progestins every second month might be alternatives to regular monthly sequential hormonal replacement therapy.

Sitruk-Ware *et al.* (1995) studied the transdermal application of steroid hormones for contraception. Estradiol is the most potent appropriate steroid for transdermal delivery and can be combined with progestins to ensure contraceptive effect. Only potent progestins could be used to achieve effective plasma level with low doses in order to maintain an acceptable small surface of transdermal delivery system. They found that transdermal delivery system changed weekly and delivering both estradiol and levonorgestrel at a daily dose of 38.4 and 28.8 $\mu g/10cm^2$ per day respectively showed ovulation suppression.

Anapliotou *et al.* (1995) evaluated the contribution of hypogonadism to the development of osteoporosis and to assessed the efficacy of new sex hormone replacement therapy regimen. They suggested that the hypogonadism seems to play an important role in the development of osteopenia-osteoporosis in thalassaemia major; continuous hormone replacement with transdermal estrogen for female or hCG for responding males best improves the bone density parameters.

Loftsson *et al.* (1995) studied fatty acids from cod-liver oil as skin penetration enhancers. The fatty acid composition of the extract was determined and it was found to

contain a vast variety of fatty acids. About 17% of the fatty acids consist of saturated fatty acids; the rest was unsaturated fatty acids. The extract was an effective transdermal penetration enhancer and this effect was found to be associated with the unsaturated portion of fatty acids.

Ditkoff *et al.* (1995) studied the impact of estrogen on adrenal androgen sensitivity and secretion in polycystic ovary syndrome. Adrenal hyperandrogenism is a common feature of patients with polycystic ovary syndrome (PCO). This may be due to enhanced adrenal sensitivity to ACTH. They concluded that estrogen was at least one factor that influenced adrenal androgen sensitivity in polycystic ovary syndrome and may help in explain the frequent finding of adrenal hyperandrogenism in this syndrome.

Ginsburg *et al.* (1995) studied the effect of acute ethanol ingestion on prolactin in menopausal women using estradiol replacement. They found that in menopausal women using transdermal estradiol, acute ethanol ingestion is associated with an increase in serum prolactin.

Wysowski *et al.* (1995) reviewed the use of menopausal estrogens and medroxyprogesterone in the United States, 1982-1982. They reported an estimated 13.6 million prescriptions were dispensed for oral menopausal estrogens in 1982, and 31.7 million in 1992, a 2-3-fold increase. In 1992, Premarin, the only oral conjugated estrogen currently approved for use, was the most frequently-dispensed brand name pharmaceutical in the United States. Dispensed prescriptions for Estraderm, a transdermal estradiol first marketed in 1986, increased from 1.5 million in 1987 to 4.7 millions in 1992. Dispensed prescriptions for oral medroxyprogesterone acetate also increased from 2.3 million prescriptions in 1982 to 11.3 million in 1992, a 4.9 fold increase. An estimated one in six to in four postmenopausal women were taking menopausal hormone in 1992. They concluded that the use of menopausal estrogens and medroxyprogesterone increased substantially over the past decade. These trends indicate that American women are widely exposed to menopausal hormone replacement therapy.

Gurwood *et al.* (1995) reported the idiosyncratic ocular symptoms associated with the estradiol transdermal estrogen replacement patch system. They suggested that ocular side effect associated with Estraderm; reported in the literature, include fluctuation in corneal curvature and variation of keratitis sicca. Clinician should be aware of the unusual symptoms that could accompany the use of estradiol estrogen replacement patch and include them among the differential diagnosis.

Rohr *et al.* (1995) studied the kinetics of new patch for transdermal administration of 17-beta estradiol. They found that this newly developed product presented a kinetically optimized transdermal 17-beta estradiol patch for hormone substitution therapy.

Lindheim *et al.* (1994) determined the effect of transdermal estrogen on insulin sensitivity in postmenopausal women and also compared this effect with changed observed with oral conjugated equine estrogens. They concluded that bimodal effect of oral equine estrogens on insulin sensitivity with an improvement occurring with the lower dose of 0.625 mg but with deterioration with the dose of 1.25 mg. They suggested that this effect may be related to a first pass hepatic portal effect in that transdermal estradiol (0.1mg), which may be equated more closely with the larger dose of oral estrogen (1.25 mg), improved insulin sensitivity. Progestins, however, appeared to attenuate the beneficial effects of transdermal estrogen and may alter the clearance of insulin.

Liu *et al.* (1994) described an experimental and theoretical evaluation of 17-beta estradiol transport in post-surgery fresh human skin in-vitro: diffusion and metabolism in each skin layer. They compared to fresh hairless mouse skin to fresh human skin appears more resistant to the stratum corneum diffusion of estradiol and were much less capable of metabolizing E2 to E1. These in-vitro results have been extrapolated to possible in-vivo human skin situation with blood vessels directly beneath the viable epidermis provided sink conditions short distance from the dermo-epidermal junction. Finally they found that the model analysis has demonstrated that there would be fewer metabolisms. Smaller amount of the transdermal metabolite (E1) would be taken up by the blood capillary due to shorter dermis path length for permanents in-vivo than in the in-vitro case using dermatomed split-thickness skin.

Prelevic *et al.* (1994) studied the effect of oestrogen and progestogen replacement therapy on systolic flow velocity in healthy postmenopausal women. They reported that the postmenopausal women had significantly lowered ejection fraction (EF), peak systolic flow velocity over the aortic valve (PVP), flow velocity integral (FVI), and mean acceleration (MA) but longer acceleration time (AT) and ejection time (ET) compared to premenopausal women. After three months transdermal estradiol significant increased in EF, PFV, FVI and MA were observed whilst AT decreased. The response in all cardiac flow indicators was a similar with added progestogen. Nevertheless the addition of progestogen does not attenuate the effect of estrogen therapy on left ventricular systolic flow velocity.

Frenkel *et al.* (1994) investigated the acceptability and skin reaction to transdermal estrogen replacement therapy in relation to climate. They found that the acceptability of Estraderm TTS was high (78.8%). Discontinuation of the treatment was found in 21.2% of the study group. The main reason for discontinuation was due to the skin reactions, which occurred in 14 of the 80 patients (17.5%). Meteorological conditions in this study did not influence the rate of skin reactions. No difference in acceptability of the treatment was found in relation to the mean temperature and mean humidity as registered in the dry desert and the humid coastal areas.

Kushida (1994) reviewed pharmacologic therapies for the prevention and treatment of osteoporosis. Vitamin D metabolites, estrogen, estrogen progestins, calcitonin and isoflavone are effective regimens for the prevention of bone loss and the treatment of osteoporosis. Since estrogen deficiency is the main cause for postmenopausal osteoporosis, estrogen supplementation is the most effective therapy for postmenopausal osteoporosis. Estrogen progestins combination therapy was the most potent therapies to increase both cortical and tubular bone mass. Vitamin D has efficacy on increasing intestinal calcium absorption and decreasing fracture rate in osteoporotic patients. Calcitonin inhibits osteoclastic bone reabsorption and also has inherent analgesic activity. Several studies have suggested that new delivery system of estrogen (transdermal) and calcitonin (Tran-nasal) is effective in osteoporosis. Recent studies revealed that the Biophosphonate and hPTH (1-34) were new potent agents for the treatment of osteoporosis. Biophosphonate would be effective to osteoporosis by reducing bone reabsorption. HPTH may have a primary action to increase bone formation.

Creatsas *et al.* (1994) studied transdermal estradiol plus oral medroxyprogesterone acetate replacement therapy in primary amenorrheic adolescents. Clinical, hormonal and metabolic aspects. They concluded that this kind of replacement therapy is efficient and safe for the treatment of amenorrheic adolescents due to gonadal dysgenesis.

Hall *et al.* (1994) assessed the effect of hormone replacement therapy on disease activity in postmenopausal rheumatoid arthritis. They concluded that this study did not show an overall effect of hormone replacement therapy on disease activity when used as an adjunct therapy in postmenopausal patients. A subgroup of patients, who had greater increments in serum E2 whilst taking hormone replacement therapy, demonstrated

improvements in some parameters of disease activity, suggesting a beneficial effect with good compliance and higher dose of hormone replacement therapy. Most importantly, in the treatment of RA associated bone loss, hormone replacement therapy could be prescribed without fear of disease flare up.

Kaplan *et al.* (1994) investigated whether treatment for prevention of osteoporosis by means of postmenopausal hormone replacement therapy and daily exercise, had any effect on rations of urinary calcium: creatinine and magnesium: creatinine. They found that hormone replacement therapy showed statistically significant lowering effects on urinary calcium: creatinine and magnesium: creatinine ratios, these return to pretreatment levels 6 months after withdrawal from hormone replacement therapy.

O'Neill *et al.* (1993) reported an Australian experience of transdermal oestradiol patches in a subtropical climate. They observed that there was, overall good efficacy and acceptability. The majority of the women found the patch very adequate in relieving symptoms of the menopause (vasomotor, genitourinary and musculo-skeletal). Most preferred the patch to oral therapy. The oestradiol patch was well tolerated and few side effects were reported. Breast tenderness and weight gain were 2 minor problems associated with its use. Skin irritation was minimal, but adhesiveness was a problem during summer months, especially with swimming or showering.

Lindsay (1993) suggested the criteria for successful estrogen therapy in osteoporosis. For maximum benefit, preventive therapy should begin as early as possible after ovarian failure begins to occur. Efforts to prevent bone loss are likely to achieve the best results when initiated prior to significant loss of bone tissue and trabecular penetration. For women with a uterus in place, a progestins usually a provided to protect the endometrium; it may given cyclically in younger women but may be given continuously in women several years past menopause. Progestins did not interfere with the effect of estrogen on skeleton, and it was possible that some progestins enhance the skeletal effects of estrogen. For patients with osteoporosis, estrogen can be used as first line therapy since in these patients they have the same skeletal stabilizing effect and reduce the risk of recurrent fracture.

Insua *et al.* (1993) reported that the hormone replacement therapy reduces the cardiovascular morbidity and mortality. They suggested that the hormone replacement therapy by transdermal route did not always modify the lipid profile, when it does changes are similar to those observed under the oral route in that the LDL/HDL ratio is

diminished but VLDL do not rises. The overall result is considered favorable, principally as a consequence of its cardioprotective.

Lindgren *et al.* (1992) evaluated the effect of transdermal sequential treatment with estradiol and estradiol/norethisterone acetate on lipoprotein metabolism. Plasma total cholesterol, low LDL and both HDL density lipoprotein were all significantly reduced both estrogen and combined phase. The findings of study by transdermal sequential hormonal treatment resulted in a lipid and lipoprotein pattern with reduced total cholesterol and LDL cholesterol in postmenopausal women.

Keller *et al.* (1992) studied the transdermal regimen for continuous hormone replacement therapy. In this study a skin patch releasing 0.05 mg estradiol and norethisterone acetate per day. They suggested that no endometrial hyperplasia was observed and there was no significant change in plasma lipids, i.e. cholesterol, triglycerides, HDL, HDL-2, HDL-3 cholesterol, LDL cholesterol and lipoproteins A1 and B. the regimen might be useful alternatives to oral continuous combined replacement therapy.

de Cecco *et al.* (1992) evaluated the endometrial response in sequential cyclic therapy and assessed with dissociated hysterectomy and histology.

Crook *et al.* (1992) compared the effect of transdermal and oral hormone replacement therapy on lipid proteins and they found that the transdermal and oral hormone replacement therapy had similar effect on lipoprotein cholesterol but different effect on triglycerides.

de Ziegler *et al.* (1992) studied the effect of luteal estradiol on the secretary transformation of human endometrium and plasma gonadotropins. They suggested that in women deprived ovarian function, administration of P only after 14 days of E2 priming prevented uterine bleeding and induced normal secretory transformations of the endometrium, but failed to suppress plasma gonadotropins.

Torres *et al.* (1992) studied the allergic contact dermatitis from nitroglycerine and estradiol transdermal therapeutic systems.

Smith *et al.* (1991) studied the effect of vehicle supplementation on total estradiol absorption from a transdermal estradiol delivery system. They suggested that the addition of ethanol to Estraderm patch increased the duration of elevated serum E2 level measured in menopausal women, thus potentially increased the effective life span of transdermal therapeutic system.

Chen *et al.* (1991) studied the transdermal permeability of l-and dl-norgestrel through human skin in-vitro at 6 skin regions with or without stratum corneum. They found that the permeation rates of dl-norgestrel through intact skin were significantly higher than those of l-norgestrel. For the skin without stratum corneum, the permeation rate and permeation amount of l-and dl-norgestrel were higher than those for the intact skin.

Ranade *et al.* (1991) studied 6 transdermal drug delivery system and they suggested that four system were available that allowed for effective absorption of drugs across the skin. The micro-sealed system was partition controlled drug delivery system that contains a drug reservoir with a saturated suspension of a drug in water miscible solvent homogenously dispersed in a silicon elastomer matrix. A second system was the matrix diffusion controlled system. The third and most widely used system for transdermal drug delivery was the membrane permeation controlled system. A fourth system recently made available, was the gradient-charged system. Additionally, advanced transdermal carriers include systems such as iontophoretic and sonophoretic system, thermosetting gels, prodrugs, and liposome's. Many drugs have been formulated in the transdermal systems and other was also examined for the feasibility of their delivery in this manner (e. g. nicotine, antihistamines, beta-blockers, calcium channel blockers, non-steroidal anti inflammatory drugs, contraceptives, anti arithmetic drugs, insulin, antiviral, hormones, alpha-interferon, and cancer chemotherapeutic agents).

Friend *et al.* (1991) studied the transdermal delivery of levonorgestrel.

Zhong *et al.* (1991) studied the influence of vehicles on human skin permeation of norgestrel and levonorgestrel in-vitro. They found that oleic acid and aqueous ethanol both enhanced synergistically the permeation of levonorgestrel through the human cadaver skin. When oleic acid and 80% ethanol was used as a co- vehicle, the flux of levonorgestrel was 0.53 microg/cm^2.h, which was 3 fold and 29 fold respectively greater than that when 80% ethanol and neat oleic acid were used as a vehicle.

Franklin *et al.* (1990) reported recently approved and experimental methods of contraception. They reviewed that the U. S. Food and Drug Administration had recently approved two contraceptive methods, and others are nearing approval. In addition several innovative approaches to contraception was under investigation. This paper described the latest information on the following methods: paragard Copper T 380A intrauterine device, cervical cap, NORPLANT, vaginal pouch, long-acting injectables, hormonal

vaginal ring, transdermal patch, experimental method male methods, inhibin, and contraceptive vaccines.

Prince *et al.* (1990) studied the effect of transdermal estrogen replacement therapy on parathyroid hormone secretion. They concluded that the effect of transdermal estrogen replacement on parathyroid secretion was completely explained by lowering of ionized calcium, causing a rise in parathyroid secretion.

Tymes *et al.* (1990) studied the in-vitro release profile of estradiol transdermal therapeutic systems by paddle method, where the patch was held in position at the bottom of dissolution vessel by sandwiching it between a watch glass and an aluminum wire mesh or Teflon screen and also by the manufacturer's paddle-over-disk method. The release profiles by both procedure were comparable and showed that approximately 10% of the labeled drug was released in 4 days.

Catz *et al.* (1990) studied the in-vitro evaluation of transdermal Levonorgestrel.

Hogan *et al.* (1990) reviewed the adverse reaction of transdermal drug delivery systems.

Fraser *et al.* (1990) determined the optimal dose of oral norethindrone acetate for addition to transdermal estradiol. They added one of three doses (0.25, 0.75, and 1.0 mg/day) of norethindrone acetate for 12 days each month to continuous, transdermal estradiol (0.05 mg/ day) determined in a prospective, randomized, multicenter study. Significant symptomatic and physiologic improvements were observed and with one exception, were not opposed by the added progestogen. Distinct redness at the site of last patch application was reported by 10% patients and faint erythema by 30%.

Chen *et al.* (1990) studied the transdermal permeability of estradiol through human skin of different body region in-vitro. They suggested that the stratum corneum acts as the rate-limiting barrier in the skin permeation of estradiol, and that the difference in estradiol permeation rates for different skin regions was mainly caused by the different extents of the barrier.

Claudy (1990) studied the hyper-pigmentation induced by UVB at the application site of estradiol. They suggested suberythemogenic UVB phototherapy to the patients to control pruritus. They found that hyper-pigmentation was more at the site of application site of estradiol. As per author, it was the first report suggesting a direct relationship between estrogen and melanin synthesis in human.

Friend *et al.* (1989) developed transdermal drug delivery of Levonorgestrel V and evaluated for their ability to co-deliver levonorgestrel and permeation enhancers ethyl acetate and ethanol in-vitro.

Adami *et al.* (1989) studied the effect of transdermal estradiol in the treatment of postmenopausal bone loss. They suggested that the bone sparing effect of estrogens might be due to only low circulating estradiol concentration, but not to supraphysiological estrone level. It seems also that estrogens exert both a straight inhibition of bone reabsorption and partial inhibition of parathyroid responsiveness.

Friend *et al.* (1989) studied the effect of enhancers on transdermal delivery of levonorgestrel. VII in-vivo. They suggested that for rabbits, a rate limiting membrane was required to control the delivery of enhancer(s) to the skin so that constant plasma levels could be maintained. The device induced mild erythema and very mild edema over 24 h exposure periods.

Friend *et al.* (1989) developed transdermal drug delivery of Levonorgestrel. IV and evaluated the membrane properties.

Tojo *et al.* (1989) developed a method for predicting steady state rate of skin penetration in-vivo. They studied the steady-state rates of penetration of ascorbic acid and estradiol across hairless mouse skin and suggested that the proposed in-vivo methods can predict the steady-state rate of penetration of these drugs across he stratum corneum in normal skin.

Loftsson *et al.* (1989) studied the effect of choline esters and oleic acid on the penetration of acyclovir, estradiol, hydrocortisone, nitroglycerine, retenoic acid and trifluorothymidine across hairless mouse skin in-vitro. Five choline esters, lauroylcholine, myristoylcholine, palmitoylcholine, stearoyl choline, oleoyl choline were evaluated as skin penetration enhancers by testing their effects on the penetration of six drugs, acyclovir, estradiol, hydrocortisone, nitroglycerine, retenoic acid and trifluorothymidine. They found that adding small amounts of choline esters and / or oleic acid to the vehicle could, significantly increase the transdermal drug delivery of the drug tested from propylene glycol vehicle system. Lauroylcholine was a better enhancer than oleic acid for the transdermal delivery of 17-beta estradiol and, in mixture, lauroylcholine and oleic acid acted as synergists giving larger enhancement of the transdermal delivery of nitroglycerine and acyclovir than when used separately.

Holdiness (1989) reviewed of contact dermatitis associated with transdermal therapeutic systems. Clonidine, estradiol, nitroglycerine, testosterone and scopolamine were utilized in such application. They found that most cutaneous reaction was limited to localized dermatitis, however, generalized systemic effects may occur.

Droesch *et al.* (1988) studied the transdermal estrogen replacement therapy in ovarian failure.

Schwartz *et al.* (1988) studied the allergic contact dermatitis from hydroxypropylcellulose in a transdermal estradiol patch.

Brown *et al.* (1988) reviewed the transdermal drug delivery of drugs. They described the theory of transdermal drug penetration, the rate of the skin, in-vitro testing, examination of currently available transdermal delivery systems, and recent developments in iontophoresis, prodrugs and penetration enhancers.

Lievertz *et al.* (1987) studied pharmacology and pharmacokinetics of estrogens via different route and suggested that transdermal patches were not subjected to a first-pass effect, provide relatively uniform serum level, and may help alleviate the problem of noncompliance.

Shah *et al.* (1986) studied the Hercon technology, for transdermal delivery of drugs was based on multi-layered laminated polymeric structure, in which a layer of vinyl chloride copolymer or terpolymer containing the drug was sandwiched between two or more layer of polymeric films. The drug was released from the device at a controlled rate by a process of diffusion through the reservoir and one of the outer layers, which can function as a rate controlling membrane.

Chetkowski *et al.* (1986) studied the biologic activity of transdermal estradiol and oral preparation. They found that both preparation lowered gonadotropins levels, decreased the percentage of vaginal parabasal cells, increased the percentage of superficial cell, and lowered urinary calcium excretion. The higher dose of oral estrogen had favorable effects on concentrations of low-density and high-density lipoproteins, but transdermal did not. They suggested that transdermal estradiol could elicit many of the desirable effects of estrogen while avoiding the pharmacologic effects of oral estrogens on hepatic protein.

Herbal Contraception:

Jainadu *et al.* (1997) studied the traditional fertility regulation among the Yoruba of South-western Nigeria. I. A study of prevalence, attitudes, practices and methods.

They found that the knowledge of traditional contraceptives was nearly universal among the Yoruba population, and the traditional contraceptives prevalence rate was 7.1%. The use of traditional contraceptives was significantly more common among uneducated women and among women aged 20 to 29 year old. Finding also revealed the existence of four main varieties of traditional contraceptives, varieties of herbal and animal products used, method of administration, and taboos against usage.

Agadjanian *et al.* (1998) performed research on women's choice between indigenous and western contraception in urban Mozambique and they suggested that women's choice between the two types of methods was determined by their sociodemographic characteristics and cultural background, access to these methods, perception of effectiveness and undesirable side effects of these methods, and restriction imposed by the providers.

Desta (1994) studied the 70 Ethiopian traditional herbal drugs for anti-fertility activity. They found total number 210 extracts from 70 traditionally used Ethiopian plants were subjected to uterogenic and anti implantation bioassay. They suggested that total of 24% of the samples had uterotonic activity and 33% exhibited anti-implantation activity.

Ye *et al.* (1994) observed the anti-fertility effects of TII and tripchloride (T4, isolated from TII) in male rats. In rats fed with TII at a dose of 10 mg/kg/day for seven weeks, the seminiferous tubules were essentially not influenced. However, most of the sperm heads along the surface of the tubular lumen were transformed from the normal sickle-shaped to round shaped, suggested a possible mutagenic action.

Juneja *et al.* (1993) studied the mouse sperm-egg interaction in-vitro in the presence of neem oil and they found that the presence of neem oil at concentration of 10, 25, and 50% caused inhibition of in-vitro fertilization in a dose dependant manner. A total 94.1% inhibition of two-cell formation and 100% inhibition of blastocyst formation from the inseminated ova were observed in 50 and 25 % neem-oil treated group respectively.

Ruan *et al.* (1991) studied the "Structure determination of marsdekoiside C a prgnane glycoside from Marsdenia koi Tsiang by column chromatograpgy and both compound mardekoiside A and B showed antifertility activity.

Raghuvanshi *et al.* (1987) reported spermicidal and contraceptive properties of Praneem polyherbal pessary they found that praneem polyherbal pessary has potent

spermicidal action on human sperm in-vitro and in-vivo. When applied in the vagina before mating, it prevented rabbits from becoming pregnant.

Sarma *et al.* (2000) studied the effect of composite root extract on histological structures of graffian follicle and endometrial epithelium in albino rat and the ovarian follicle showed structural disparity in thecal cells and granulose cells and formation of zona pellucida. In the uterus, the endometrial epithelium on the luminal surface showed pseudotratification, vacuolation of the cells and irregular desquamation from the stroma. Infiltration of a large number of polymorphs in the endometrial stroma and necrosis of endometrial gland tissues indicated structural and functional aberrations of the uterus.

Benie *et al.* (1990) performed the extraction of Combretodendron africanum bark, as an antifertility agent. I: estrogenic effects in vivo and LH release by cultured gonadotropic cells. They found that the Combretodendron africanum extract was shown to complete with estradiol and with progesterone on uterine receptors. Consequently, it was thought to contain substances exhibiting estrogenic potency. The effect of the extract on LH-RH-induced release were amplified, the extract itself appeared as to be a potent secretagogue not requiring LHRH receptors. It follows that the active molecules contained in Combretodendron africanum extracts might be different from classical steroid estrogens.

RESEARCH ENVISAGED AND PLAN OF WORK

Basic Principle of Hormone replacement therapy

17-beta estradiol, conjugated equine estrogens, esterified estrogens, and estradiol constitutes postmenopausal replacement therapy, all of which are in clinical use as oral preparation. Non-oral route-matrix and reservoir patches, gels have already been developed for estradiol. Similarly intravaginal administration of estriol and estradiol has been reported. Daily dose of 1 mg estradiol valerate or 25 μg estradiol delivered via a transdermal patch or 0.5 mg in gel or 0.3 mg conjugated equine estrogens are often sufficient to alleviate climacteric symptoms. Bone reabsorption may be effectively reduced and bone mineral density maintained by 1 mg estradiol or 25 μg transdermal estradiol. Maximal bone sparing dosages are 2 mg estradiol, 50 μg transdermal estradiol; 0.625 mg conjugated equine estrogens, 1.25 mg estrone. Estradiol predominantly used for the prevention and or treatment of urogenital symptoms, has no bone sparing effect at the doses in clinical use. Non-oral administration of estradiol may be superior in diabetic women and those with hypertriglyceridemia due to the different metabolism, which does not mainly involve the hepatic, first pass effect. Epidemiological data do not support any preference of oral versus non-oral routes of administration regarding side effects such as venous thromboembolism. Progesterone e.g. natural progesterone, derivatives structurally related to progesterone and testosterone, respectively is necessary for endometrial protection. Sequential use of progesterone for at least 10 days per month, preferably 12-14 days abolishes the increased incidence of endometrial hyperplacia, which is likely to develop with unopposed use of estrogen. Observational studies do not suggest any superiority of given progesterone regarding cardiovascular risk prevention of osteoporosis, and cognitive function in postmenopausal women on estrogen replacement therapy. Tibolone, a derivative of norethindrone, is yet another option for replacement therapy. The recommended dose for treatment of climacteric symptoms and prevention of bone loss is 2.5 mg. Controlled clinical studies do not suggest that this compound is superior in achieving ammenorrhea compared with continuous combined estrogen progesterone replacement therapy, as available data are inconsistent. In early menopause, the sequential use of progesterone in conjunction with an estrogen is the preferred treatment option. With advancing postmenopausal age, either continuous combined replacement or tibolone may be choices in case withdrawal bleeding is no longer

acceptable for women. However, there are no rigid age limit when to change treatments, the selection of which largely influenced by preference of the individual's acceptance of withdrawal bleeding.

Levonorgestrel is a potent progestogen and has been widely prescribed as a contraceptive steroid for female fertility regulation. But when progestin alone was used as a contraceptive, it produced various untoward effects like episodes of irregular; unpredictable breaks through bleeding within first year. These most frequently encountered side effects are the major reasons why women discontinued their use. The possibility of adverse effects of progestins on plasma lipoprotein and long term effects on thrombosis and other disorder have not been investigated to the same extent with combined estrogen-progestins preparations. Progestins alone are less efficacious than combination oral contraceptives.

The most frequently used contraceptive agent in the United States are combination oral contraceptives containing both estrogen and progestins. They are highly efficacious with theoretical effectiveness generally considered to be 99.0% and use effectiveness of 97% to 98%. Phasic preparations were developed in the 1980's, largely reduce the dose of progestins in oral contraceptives, when it was recognized that these oral contraceptives might have untoward cardiovascular effects.

One might reasonably conclude that the most widely used preparations to date owe their effectiveness in inhibiting ovulation to the estrogenic component and that the progestins serves the major purpose of ensuring that withdrawal bleeding will be prompt, brief in duration and essential pharmacologically. Even if ovulation were not stopped, it is easy to imagine that the contraceptive agents could interfere with impregnation by their direct action upon genital tract by showing direct effect on endometrium and cervix.

Growing evidence demonstrates that the transdermal route offers several potential advantages over conventional routes for systemic medication, avoidance of first pass elimination, extended duration of therapeutic activity with reduced side effects and patient compliance. Research has been persuaded to enhance the permeability of drug across the stratum corneum and to achieve higher systemic concentration of drugs. (Barar, 1989 and Tripathi, 1994)

Several types of transdermal therapeutic systems, which utilize the rate controlled drug delivery technologies to modulate the transdermal systemic delivery of therapeutic agent have been successfully developed and commercialized.

Although a few vesicular systems like liposomes and niosomes have been studied for contraception through transdermal routes, but their unstable nature limits their use in TDDS. Therefore, in order to increase the stability of liposomes, concept of proliposomes has been proposed. In a similar approach some of the researchers studied niosomes, which exhibit superior stability and free from other limitations of liposomes like fusion, drug leakage etc.

The literature survey reveals that no investigation has been reported to date on the use of proniosomes to achieve a combined delivery of estradiol plus levonorgestrel and ethinyl estradiol plus levonorgestrel from the same unit of provesicular transdermal therapeutic system.

Therefore it was thought worthwhile to incorporate proniosomes in TDDS for contraception and hormone replacement therapy. Optical anisotropic proniosomes seems to convert into the niosomes *in situ* by absorbing water from the skin. In addition to providing the controlled systemic transdermal delivery of contraceptive agents, the proposed system may have some added advantage like;

- Greater stability
- Higher entrapment efficiency; and
- Greater membrane permeability and enhanced penetration.

Plan of work

The proposed work was carried out under the following plan:

1. Identification and characterization of selected drugs and surfactants
2. Preformulation studies:
   (i) I.R. analysis
   (ii) Solubility determination in different solvents
   (iii)Partition coefficient determination
3. Preparation and characterization of Proniosomes:

(i) Preparation of Proniosomes without drugs

(ii) Preparation of Proniosomes with drugs

(iii)Microscopic study of Proniosomes

   (a) Phase contrast microscopy
   (b) Polarized microscopy

(iv) Characterization of niosomes derived from proniosomal systems;

(a) Study and mechanism of formation of proniosomal system

(b) Spontaneity of niosomes formation

(c) Size distribution study

(d) Drug entrapment efficiency

4 (i) Preparation of proniosomes formulation optimization using following process variables:

(a) Amount of drug in the film

(b) Surfactant

(c) Alcohols

(d) Sonophorosis

(e) Composition of Film

(ii) Evaluation of prepared proniosomes for in-vitro drug release and percutaneous absorption studies using albino rat skin and effect of following factors on release rate:

(a) Amount of drug in the film

(b) Surfactant

(c) Alcohols

(d) Sonophorosis

(e) Composition of Film

5. Stability studies of selected formulations

(a) Temperature

(b) Ageing effect

6. *In vivo* evaluation of selected products on the laboratory animals for the following parameters.

(a) Endometrial effects

(b) Vaginal cornification study

(c) Effect on vaginal thickness

(d) Effect on formation of corpus luteum

(e) Growth response of uterus with estradiol, ethinylestradiol and levonorgestrel

(f) Growth response of uterus from formulation containing estradiol and levonorgestrel

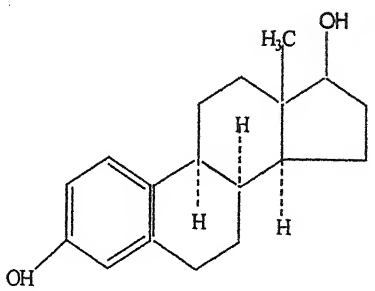
(g) Growth response of uterus from formulation containing ethinylestradiol and levonorgestrel

(h) Effect on lipid profile

Chapter-2

ANALYTICAL PROFILE OF DRUG SELECTED

Analytical profile of Drugs Selected



Estradiol

It is β Estradiol, estra-1, 3, 5 (10) triene 3,17 β diol, Dihydro-follicular

Molecular Formula: $C_{18}H_{24}O_2$

Molecular Weight: 272.39

Description:

It is a white to creamy or slightly yellowish white, odorless, crystalline powder. It is obtained as hemihydrates, fine needles from methanol and water.

Solubility:

Practically insoluble in water, 1 part soluble in 28 of ethanol, 1 in 150 of ether, 1 in 5 of acetone, 1 in 4 of dioxane and 1 in 435 of chloroform. Soluble in vegetable oils and solution of alkali-hydroxide.

Packing and Storage:

Stored in non-metallic airtight containers, protected from light.

Pharmacokinetics:

Estradiol is mainly metabolized in the liver, the major metabolites being estrone and estriol and their conjugates, which are considerably less potent, that estradiol. The bulk of estradiol is excreted in the urine as glucuronides and sulphates, although some enterohepatic re-circulation may occur. The plasma elimination half-life of estradiol is approximately 1 hour irrespective of the route of administration and the metabolic plasma clearance rate is between 650 and 900 L/day/m^2.

Pharmacodynamics:

17β-estradiol is the predominant estrogen produced by the ovaries in premenopausal women. Administration of transdermal estradiol to postmenopausal women (in dosages of 0.05 to 0.2 mg/day) elevates plasma estradiol concentrations into

the range observed in premenopausal women at the early to mid follicular stage. The increased plasma estradiol concentrations, plasma concentrations of follicular stimulating hormone (FSH) and leuteinising hormone (LH) are decreased and vaginal cytology is converted to pattern resembling that found in premenopausal women with improvement of the maturation and karypyknotic indices. Bone resorption is inhibited as evidenced by a reduction in the urinary ratios of calcium and hydroxyproline to creatinine and an increase in bone mineral density.

Adverse Effects:

Oestrogen gives raise to side effects, which are related to their oestrogenic, metabolic effects. There may be sodium and water retention with oedema, weight gain, tenderness and enlargement of breast, change in libido, menstrual disorder and withdrawal bleeding, alteration in liver function, jaundice, gallstone, depression, headache, migraine, dizziness, hypercalcemia, endometrial proliferation, complex effects on cardiovascular system.

Identification test:

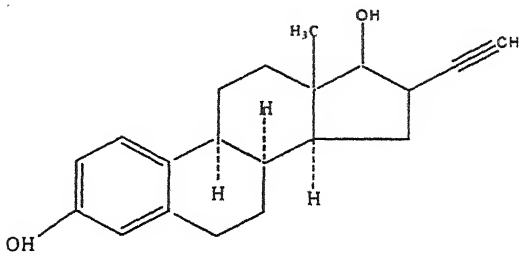
1. Heat 0.05 mg with 1.0 ml of a 2.5% w/w solution of β-naphthol in sulphuric acid for 2 minutes at 100°C, cool and add 1.0 ml of water, an orange yellow color is produced which changes to red when the solution is heated for 90 seconds at 100°C.
2. Dissolve 0.05 g in 5.0 ml mixture of equal volumes of acetone and sodium hydroxide solution and add gradually with vigorous shaking 0.5 ml of benzoyl chloride, a precipitate is formed which, after re-crystallization from mixture of equal volumes of acetone and water and drying at 105°C, melts at about 192°C.

Therapeutic Uses and Administration:

Estradiol is a synthetic estrogen with active and uses similar to other estrogens. Estradiol is frequently used as the estrogenic component of combined oral contraceptive preparations. A daily typical dose is 20 to 50 μg.

1. In menopause and postmenopausal symptoms doses of 10 to 50 μg daily.
2. For the treatment of female hypogonadism, 50 μg has been given daily for 14 consecutive days, followed by progesterone for next 14 days.
3. For the palliatives treatment of malignant neoplasm of prostate, doses of 1.5 to 3.0 mg daily.
4. Also used in combination with progestogens for disorder of menstruation.
5. With antiestrogens cyproterone it is used for severe acne.

Analytical profile of Drugs Selected



Ethinylestradiol

It is 17α-Ethinylestra-1, 3,5(10)-triene-3, 17 β diol. 19 Nor-17α-pregna-1,3,5(10)-triene-20 yne-3, 17 β diol. 17α-Ethinyl-1, 3,5(10)-estratriene-3, 17 β diol.

Molecular Formula: $C_{20}H_{24}O_2$

Molecular Weight: 296.41

Description:

It is a white to creamy or slightly yellowish white, odorless, crystalline powder. Hemihydrates, fine needles from methanol & water.

Solubility:

Practically insoluble in water, 1 part soluble in 6 of ethanol, 1 in 4 of ether, 1 in 5 of acetone, 1 in 4 of dioxane and 1 in 20 of chloroform. Soluble in vegetable oils and solution of alkali-hydroxide.

Packing and Storage:

Stored in non-metallic airtight containers, protected from light.

Pharmacokinetics:

Estradiol is rapidly well absorbed from the gastrointestinal tract. The presence of ethinyl group at C-17 position greatly reduces hepatic first pass metabolism compared with oestradiol enabling the compound to be much more active by mouth, but there is some initial conjugation by the gut wall and the systemic bioavailability is only about 40%. Ethinylestradiol exist in blood plasma proteins such as serum albumin but not Sex Steroid Binding Globulin due to their size and lipophilic nature. The elimination half-life has been reported up to 13-17 hrs, and biotransformation route via hydroxylation and subsequent formation of corresponding 2 and 3 methyl esters, excretion via the kidney with some appearing also in the feces.

Pharmacodynamics:

The hypothalamic gonadotropin-releasing hormone (FSH-LHRH) is inhibited by oestrogen, which leads to suppression of FSH-LH release from the anterior pituitary and ovulation is stopped.

Adverse Effects:

Estrogen gives rise to side effects, which are related to their oestrogenic, and metabolic effects. There may be sodium and water retention with oedema, weight gain, tenderness and enlargement of breast, change in libido, menstrual disorder and withdrawal bleeding, alteration in liver function, jaundice, gallstone, depression, headache, migraine, dizziness, hypercalcemia, endometrial proliferation, complex effects on cardiovascular system.

Identification test:

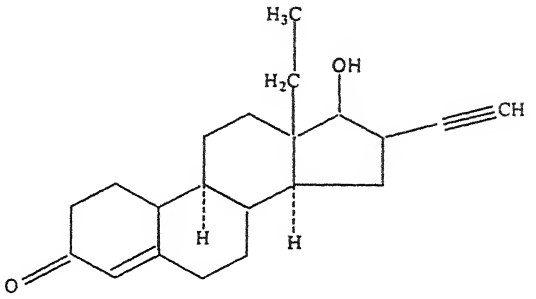
1. The infrared absorption spectrum is concordant with the reference spectrum of ethinylestradiol.
2. Absorbance of 0.01% w/v solution in ethanol (95%) at about 281 nm, 0.69 to 0.73.
3. Dissolve about 1.0 mg in 1.0 ml of sulphuric acid; an orange colour develops which exhibits a greenish fluorescence when examined under U. V. light (365nm). Add the solution to 10 ml of water, the colour changes to violet and violet precipitate is produced.

Therapeutic Uses and Administration:

Ethinylestradiol is a synthetic estrogen with activity and uses similar to other estrogens. Ethinylestradiol is frequently used as the oestrogenic component of combined oral contraceptives preparation. A daily typical dose is 20 to 50 μg.

1. In menopause and postmenopausal symptoms its daily dose is 10 to 50 μg.
2. For the treatment of female hypogonadism, 50 μg has been given daily for 14 consecutive days, followed by progesterone for next 14 days.
3. For the palliatives treatment of malignant neoplasm of prostate, doses of 1.5 to 3.0 mg is given daily.
4. Its combination with progestogens is used for disorder of menstruation.
5. With antiestrogens cyproterone used for severe acne.

Analytical profile of Drugs Selected



Levonorgestrel

It is (+) 13 β ethyl-17 β hydroxy, 18, 19, dinor-17 pregna-4 ene-20yne-3one. 1, 19-D, norpregn-4 ene-20 yne-3one, 13 ethyl-17 hydroxy.

Molecular Formula: $C_{21}H_{28}O_2$

Molecular Weight: 312.44

Description:

It is a white or nearly white, practically odorless, crystalline powder. Crystalline powder.

Solubility:

Practically insoluble in water, slightly soluble in alcohol (95%) acetone, in methanol, and in ether; soluble in 1 in 45 of chloroform.

Packing and Storage:

Stored in well-closed containers, protected from light.

Optical Isomers:

(+) Isomer of levonorgestrel is norgestrel, which is half as potent on a weight basis as is levonorgestrel.

Pharmacokinetics:

The 19-non steroids have good oral activity because the ethinyl group substituent at C-17 position significantly slows hepatic metabolism. In the plasma, norgestrel binds to sex steroid binding globulin and albumin. The total binding of all these synthetic compounds to plasma proteins is extensive i.e., 90% or more. After oral administration, the peak plasma level is achieved after 4 hrs. The plasma half-life is about 4 to 6 hrs for levonorgestrel. The drug primarily metabolized by hydroxylation and conjugation with sulphate and glucuronide. About 60% of administered dose is excreted from urine and 40% eliminated in feces.

Pharmacodynamics:

Progestins only prevent ovulation 70 to 80 % of cycles, largely by slowing the frequency of GnRH pulse generator. The blunting of LH and FSH pattern is observed in women receiving these drugs. Another mechanism is thickening of cervical mucous to decrease sperm penetration and endometrial alteration that impair implantation. These are thus thought to contribute significantly to their efficacy.

Adverse Effects:

Progestogen only should be used with care in women with heart disease, malabsorption syndromes, liver dysfunction including recurrent cholestatic, or a history of jaundice in pregnancy. Other suggested cautions for progesterone only contraceptives include diabetes mellitus, hypertension, migraine and thrombo-embolic disorders.

Identification test:

1. The infrared absorption spectrum is concordant with the reference spectrum of levonorgestrel.
2. Absorbance of 0.01% w/v solution in methanol at an absorption maximum of 240 nm is about 0.54.
3. On adding 2.0 ml of ammonia to a mixture of 0.3 ml of 0.5% w/v ethanol (95%) solution of 1, 3, dinitro benzene containing 2.0 mg drug and 0.1 ml benzalkonium chloride solution, a pink colour is immediately produced.

Therapeutic Uses and Administration:

Progestogens are used together with estrogens in contraceptives. They are used, often with estrogens in functional uterine bleeding, dysmenorrhoea, amenorrhoea, premenstrual tension and endometriosis. They are used alone in palliative management of endometrial carcinoma.

Dose of Drugs:

Estradiol	----	daily dose is 20 to 50 μg
Ethinylestradiol	----	daily dose is 20 to 50 μg
Levonorgestrel	----	daily dose is 30 to 37.5 μg

Table 2.1: Pharmacokinetics Data of drugs

Parameters	Estradiol	Ethinylestradiol	Levonorgestrel
Availability-Oral	Poor	51%±9 %	94% intravaginal, 74±16%
Biotransformation	Hepatic first pass Metabolism	Hepatic first pass Metabolism	NO, Hepatic first pass Metabolism
% Urinary Excretion	90%	1-5%	52%±8 %
% Bound Plasma	50-80%	95-98%	37%±7 %
Clearance (ml min^{-1} Kg^{-1})	---	5.4%±2 %	1.5%±0.6 %
Volume of Distribution (lit/Kg)	--	3.5%±1.0 %	1.7%
Half-Life (hrs)	20 min	10±6	15±3
Effective Concentration	---	---	max. 3-5 ng/ml
Toxic Level	---	---	0.2 mg/ml

ANALYTICAL METHODS.

Several methods have been reported in the literature for the estimation of both drugs. Some are reported as follows.

Walash *et al.* (1985) applied Zimmermann reaction for the determination of steroids. The steroids were extracted from powdered tablets with ethanol and extracts were evaporated to dryness and residue was dissolved in t-butyl alcohol-cyclohexane (9:1). Steroids in a portion of this solution were deethylated by treatment with Na-t-butoxide and resulting 17-oxo-steroids were determined after condensation with 1, 3-dinitobenzene (I) or 1, 3, 5 trinitrobenzene (II). The absorbance of the products was measured at 512 or 475 nm against a reagent blank with use of I & II respectively. This reaction was used for the determination of Ethinylestradiol, norgestrel, mestrenol and norethisterone.

Dohji *et al.* (1985) developed an HPLC method for the measurement of estradiol and estriol in biological fluid. Estradiol and estriol from serum of pregnant women were adsorbed on the pre-treatment column, hydrophilic components such as protein and carbohydrates were washed through the column with 0.05-M phosphate buffer of pH 8.5. The estrogen were then eluted on HPLC column by aqueous 65% acetonitrile and separated by a reverse phase mechanism. The fluorescence detection at 320 nm (with

excitation at 220 nm) gave rectilinear response in the range of 0 to 40 ng/ml of either analyte.

Gang *et al.* (1992) determined oestradiol in oestradiol transdermal delivery system by HPLC method using stainless steel column (15 cm X 6 mm) of Zorbox C-18n with aqueous 75% methanol as mobile phase (1 ml min^{-1}) and detection at 280 nm.

Matlin *et al.* (1985) reported HPLC analysis of steroid esters of norethisterone and levonorgestrel. The potential injectables contraceptive in CH_2Cl_2 were subjected to HPLC column packed with hypersil or hypersil ODS with acetonitrile CH_2Cl_2 or aqueous methanol as mobile phase (2 ml min^{-1}).

Bai *et al.* (1987) analyzed norgestrel (I) and ethinylestradiol (II) tablets by CH_2Cl_2 methods using a column (15 cm X 2.1 mm) of nucleosil C–18 (5 μm) with aqueous 53% methanol as mobile phase I & II were simultaneously detected at 270 nm.

Xiang *et al.* (1987) determined spectrophotometrically norgestrel (I) and ethinylestradiol (II) in compound tablets without prior separation in ethanolic medium. A powdered sample containing 0.6 mg of ethinylestradiol (II) was dissolved in 20 ml of anhydrous ethanol. The mixture containing 0.5 mg of (I) was dissolved in 50 ml of anhydrous ethanol and heated on water bath at 60°C for 30 min and filtered, and then the absorbance was measured at 276 and 279 nm respectively.

Lane *et al.* (1987) determined norgestrel (I) and ethinylestradiol (II) in tablets by HPLC methods using C-18 column (5 cm X 4.5 mm; 5μm) with H_2O-THF-methanol (13:5:2) as the mobile phase (2.1 ml min^{-1}) and 230 nm as detection range.

Reif *et al.* (1987) used automated stability indicating HPLC assay for Levonorgestrel (III) and ethinylestradiol (II) in oral contraceptives. The mixture of I & II and III were separated on C-18 or C-18 silica and mobile phase of H_2O: acetonitrile: methanol (9:7:3). (I) was detected fluorometrically at 310 nm (excitation at 210 nm) and II and III were detected by measuring at 240 nm.

Nielene *et al.* (1988) reported urine analysis of ethinylestradiol by liquid chromatography using 10 μm PRP-I with elution by using 70% methanol and 25-40 μm Ag-loaded sepharon oxime 1000 with elution by using acidified aqueous 15% acetonitrile. The HPLC column was a glass cartridge (10 cm X 3 mm) of chromosphere (5 μm) equipped with a guard column (10 cm X 2 mm) of chromosphere C-18 (5 μm) with gradient elution from aqueous 15 to 50 % acetonitrile and dual wavelength detection at 240 nm and 280 nm.

Lee *et al.* (1988) reported a method for the determination of ethinylestradiol and norethisterone in a single specimen of plasma by automated HPLC and subsequent radioimmunoassay.

Levonorgestrel and ethinylestradiol have been estimated by HPLC method using stainless steel column packed with stationary phase LC (I), (b) a mixture of acetonitrile and water (55:45) as mobile phase and detection wavelength was 241 nm for norgestrel and 305 nm for ethinylestradiol.

Ahmed *et al.* (1992) introduced cyclodextrin-bonded phase for liquid chromatographic separation and analysis of ethinylestradiol and levonorgestrel in oral contraceptive.

Gopaghose (1993) separated ethinylestradiol and norgestrel from oral contraceptives and determined by reverse phase HPLC.

Preedi and Aitken (1960) used sulphuric acid fluorescence following column chromatography for the determination of urinary estrogen.

Brown and Bould (1960) developed colorimetric estimation procedure for estriol, estrone and estradiol 17-beta using 2% quinol in 65% H_2SO_4. The intensity of color produced was measured at 430, 516 and 552 nm respectively.

Novakovid *et al.* (1991) used simultaneous capillary gas chromatographic determination of cyperoterone acetate and ethinylestradiol in pharmaceuticals. They used CP-Sil-5-CB with temperature from 210°C to 270°C at 0.2° min^{-1} for 2.5 min and 30° $min^{-1}$for 5 min; hydrogen was used as carrier gas with flame ionization detector.

Dabrowska *et al.* (1989) separated and determined ethinylestradiol and norethisterone acetate by HPLC on a Pye Unichem apparatus. The reaction times were tabulated for both on a column (12.5 cm X 4.6 mm) of Nucleosil 5C8 (10μm) or Partisil OSD 10 or column (25 cm X 4.6 mm) of Lichrosorb RP (10μm) with aqueous 70% methanol as mobile phase (1 ml min^{-1}) and detected at 230 nm.

Liu and Ma (1992) reported simultaneous determination of norethisterone and ethinylestradiol in tablets by HPLC on a column (7.5 cm X 4.6 mm) of Nucleosil C-18 (5μm) with aqueous 60% methanol as mobile phase (1 $mlmin^{-1}$) and detection at 210 nm (internal standard was norgestrel).

Carignan *et al.* (1985) reported reproducible simultaneous determination of estrogen and progestins in oral contraceptives; crushed tablets were mixed with a solution of Valerophenon (I) in aqueous 80% methanol (as internal standard0) using a column of

(25 cm X 4.6 mm) of Zorbox OSD. The steroids were eluted with aqueous 60% or 70% acetonitrile and detected at 210 nm.

Milligramme amounts of monosubstituted acetylenes, e.g. propyne norethisterone acetae and ethinylestradiol were titrated in propane, 2-ol containing piperidine as catalyst with 10mM. $AgNO_3$ in propane 2-ol. The end point was detected potentiometrically with use of a Rodelkis OP-S-711 Ag_2Selectrode Vs a SCE. It was titrated with aqueous 1M $AgNO_3$ in a medium of 67% ethanol of pH 9.2-to 9.8 borate buffer.

A tablets of ethinylestradiol was added to 1 ml H_2O and 1 ml of 4-t-pentyl phenol solution (as internal standard) and analyzed by HPLC on a column (25 cm X 4.6 mm) of Lichrosorb RP-18 (5μm) with aqueous 47% acetonitrile containing 0.1mM, Na-Acetate (pH 9.5) as mobile phase (1.7 ml min^{-1}) and electrochemical detection with use of vitreous carbon electrode at +0.85 V Vs Ag-AgCl reference electrode.

DRUG ANALYSIS

Method selected for the determination of estradiol and levonorgestrel

For the present investigation, slightly modified USP, NF, 1995 spectrophotometric method was selected on account of its specificity, sensitivity, simplicity, reproducibility, feasibility, rapidity, and accuracy.

Determination of λ_{max} for estradiol and levonorgestrel:

10 mg each of estradiol and levonorgestrel were accurately weighed and transferred in two separate 100 ml of volumetric flasks containing 40 ml of PEG 200. The drug was dissolved by gentle shaking and volume made up to mark with distilled water so that resulting solution contained of 40% v/v PEG 200 as stock solution A. Then 10 ml of this solution was transferred into a 100 ml of volumetric flask and volume made upto the mark with 40% v/v PEG 200 to make standard stock solution B of 10 µg/ml concentration. In order to determine the λ_{max} for estradiol and levonorgestrel, standard stock solution B was scanned between 220 to 320 nm using Shimadzu 1700 UV Spectrophotometer.

Determination of λ_{max} for ethinylestradiol and levonorgestrel:

10 mg each of ethinylestradiol and levonorgestrel were accurately weighed and dissolved in two separate 100 ml of volumetric flasks containing 40 ml of PEG 200. The further were made similarly as discussed above. To make standard final stock solution of 10 µg/ml concentration in 40% v/v PEG 200. In order to determine the λ_{max} for ethinylestradiol and levonorgestrel the final standard stock solution was scanned between 220 to 320 nm using Shimadzu 1700 UV Spectrophotometer. The scanned graph (λ_{max}) of estradiol, ethinylestradiol, and levonorgestrel are reported as figure-2.1, 2.2, and 2.3.

Preparation of standard curve of estradiol, ethinylestradiol, and levonorgestrel in 40%v/v PEG 200

Procedure:

From the stock solution B the aliquots of 0.1, 0.2, 0.3, 0.4, ..., 1.0 ml were withdrawn into a series of 10 ml of volumetric flasks and volume was made upto 10 ml with 40% v/v PEG 200 to prepare standard solutions containing 2.0 µg/ml, 4.0 µg/ml, ..., 20.0 µg/ml of drug. These solutions were analyzed spectrophotometrically using Shimadzu 1700 UV Spectrophotometer. The absorbance of each solution was noted at 280nm for estradiol, 281 for ethinylestradiol and 247 for levonorgestrel and plotted

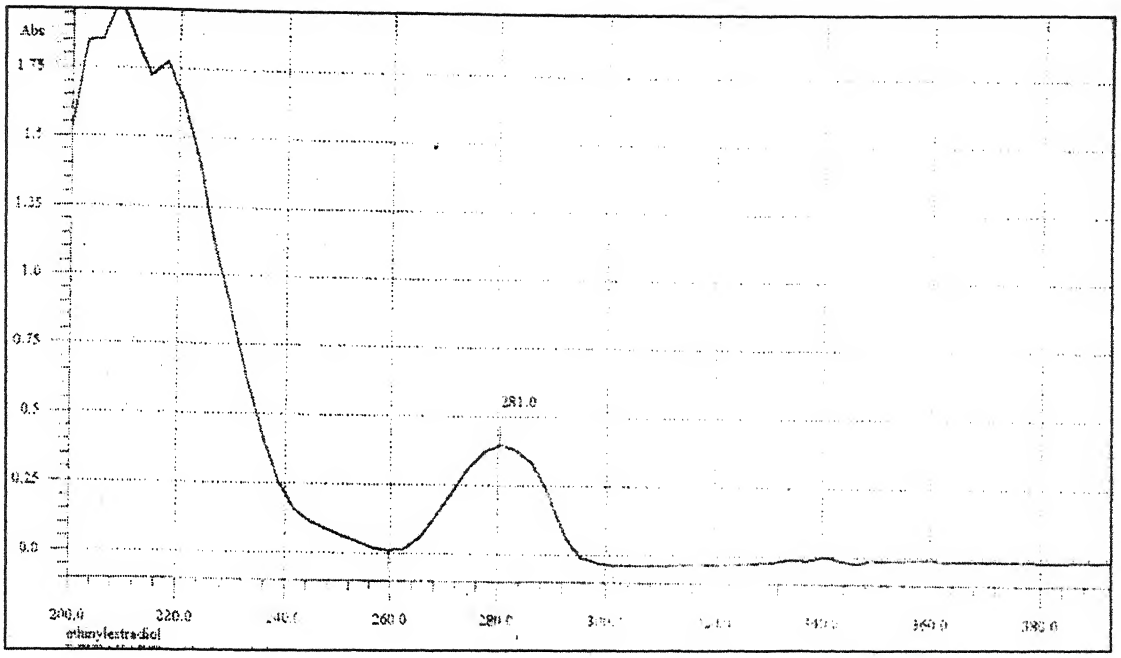


Figure-2.1: Scanned Graph of estradiol

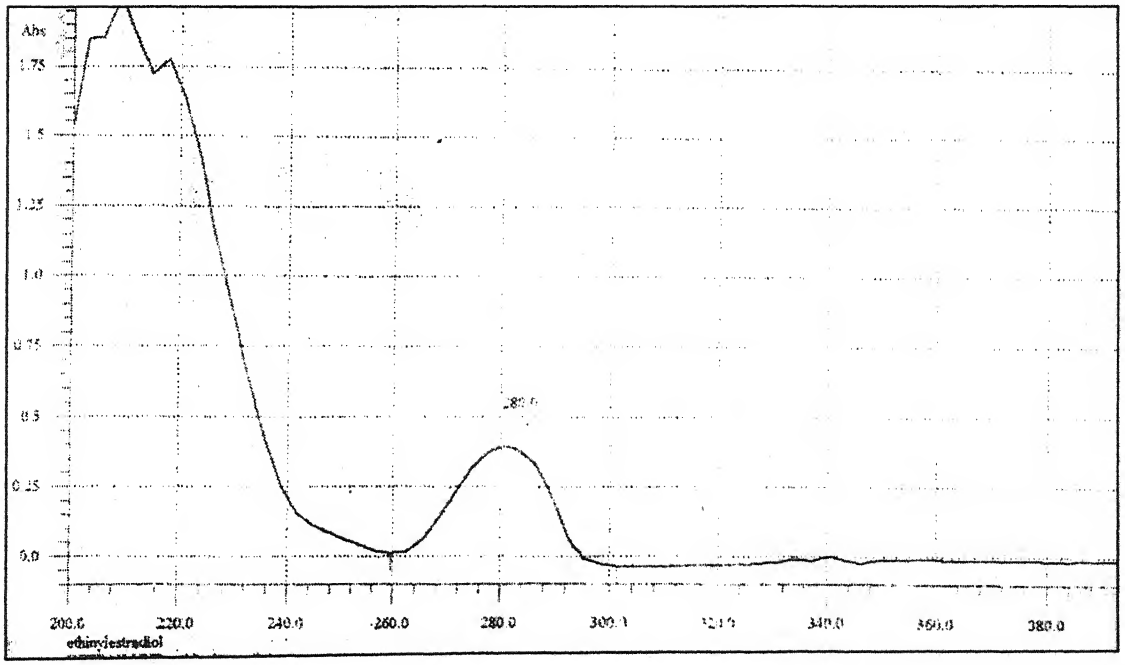


Figure-2.2: Scanned Graph of ethinylestradiol

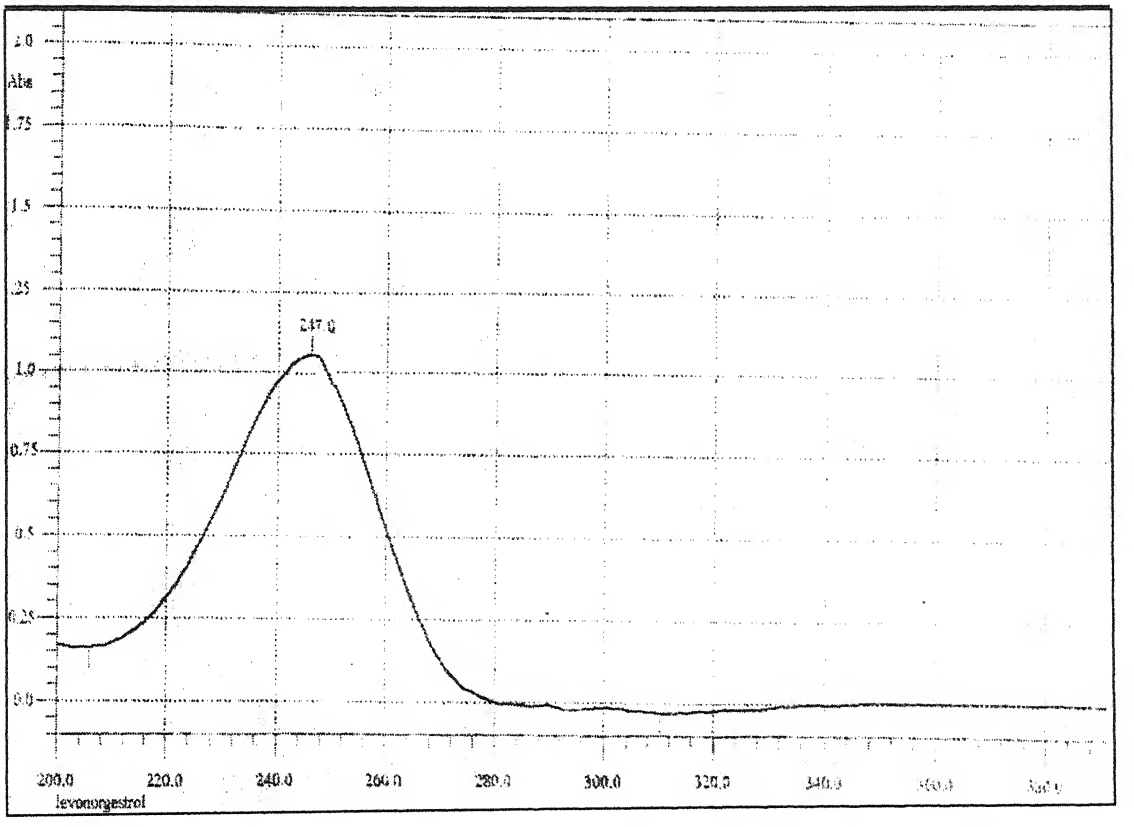


Figure-2.3: Scanned graph of levonorgestrel

against concentration to get the standard curve of estradiol, ethinylestradiol and levonorgestrel in 40% v/v PEG 200. These standard curves were linearly regressed and statistical parameters related to it were derived. The observations are recorded in table 2.2, 2.3 and 2.4 and graphically shown in figure 2.4, 2.5 and 2.6.

Simultaneous determination of component drugs in the combined formulation:

In the present work the combined formulations of either estradiol or ethinylestradiol were prepared with levonorgestrel. Therefore, methods were developed to analyze the component drugs i.e. ethinylestradiol and levonorgestrel in one combination and estradiol and levonorgestrel in other combined formulation simultaneously by the absorption method. For simultaneous determination of drugs in combined formulation the UV absorption method through multicomponent mode was developed. The principle of method is as follows:

Multicomponent quantitation mode is the mode in which the concentration of each constituent component is determined by using the absorption spectrum of the mixed sample with pure standards or standards made up of multiple constituent components. There are many advantages of multicomponent quantitation mode i.e.,

1. In a mixed sample with upto 8 constituents components can be quantitated.
2. In addition to using pure samples of each constituent component as the standards samples, a mixed sample in which the concentration of each constituent component is known may also be used.
3. The effects of interference among the various constituent components can be minimize by using a mixed sample as the standard sample.
4. The standard sample data can be saved with the measurement parameters in memory. In addition, a spectrum saved in memory can be used as the standard sample of unknown sample data. However, unlike other parameter files, these can only be saved one at a time.
5. The measurement wavelengths can be set at uniform intervals or randomly set. One can freely set the wavelengths to improve measurement accuracy.

Principle:

The absorbances of two solutes in the solution are additive provided there is no reaction between the two solutes. We may write,

$$A\lambda_1 = \lambda_1 A_1 + \lambda_2 A_2$$

and $$A\lambda_2 = \lambda_2 A_1 + \lambda_2 A_2$$

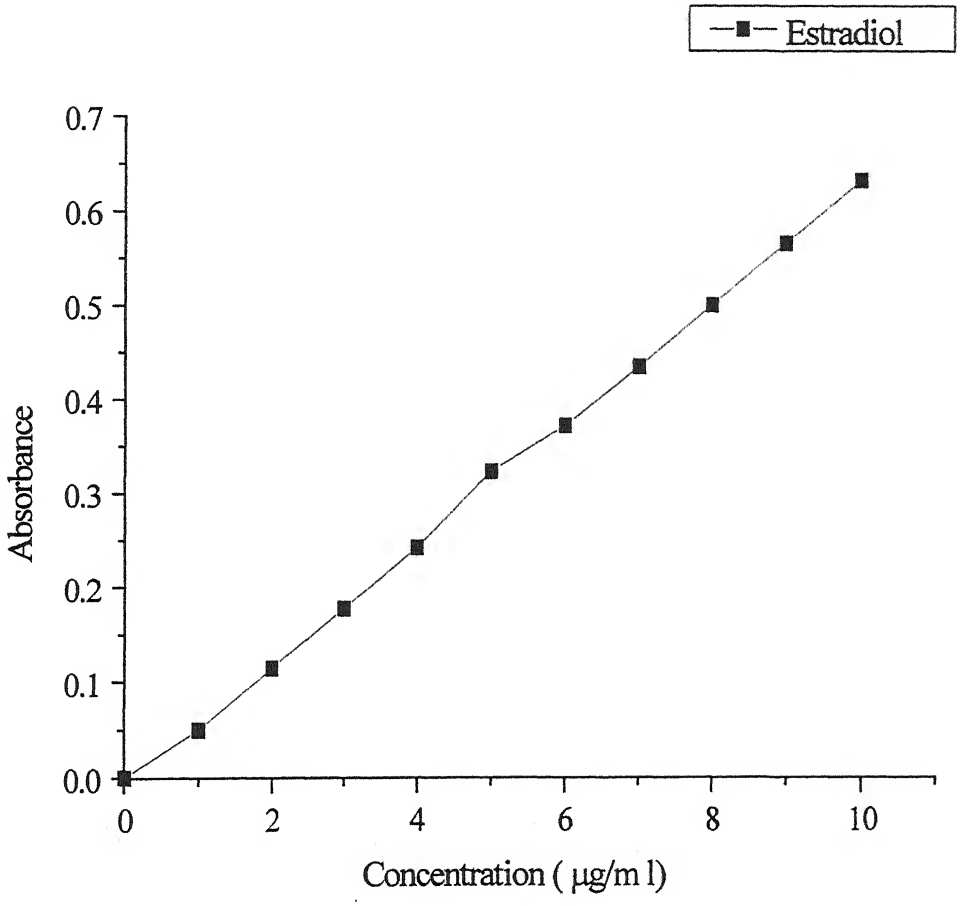


Figure 2.4: Standard Curve of Estradiol in 40% PEG-200 at 280 nm

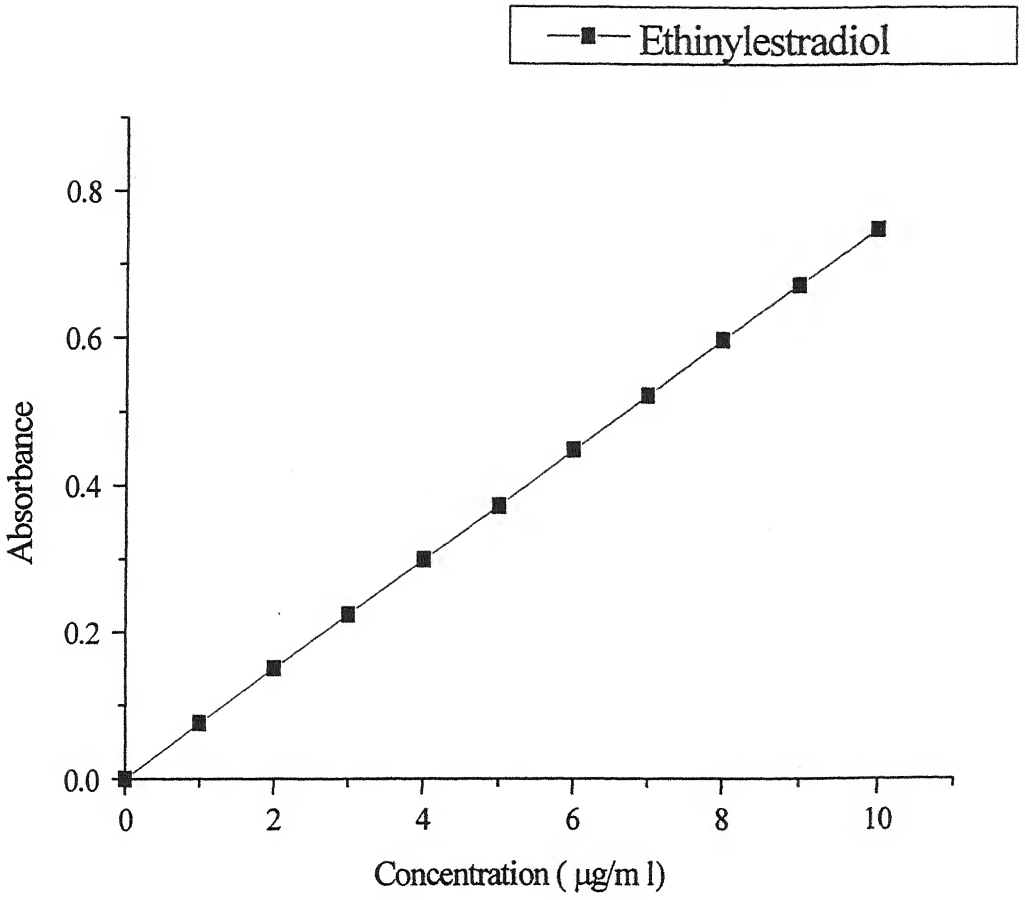


Figure 2.5: Standard Curve of Ethinylestradiol in 40% PEG-200 at 281 nm

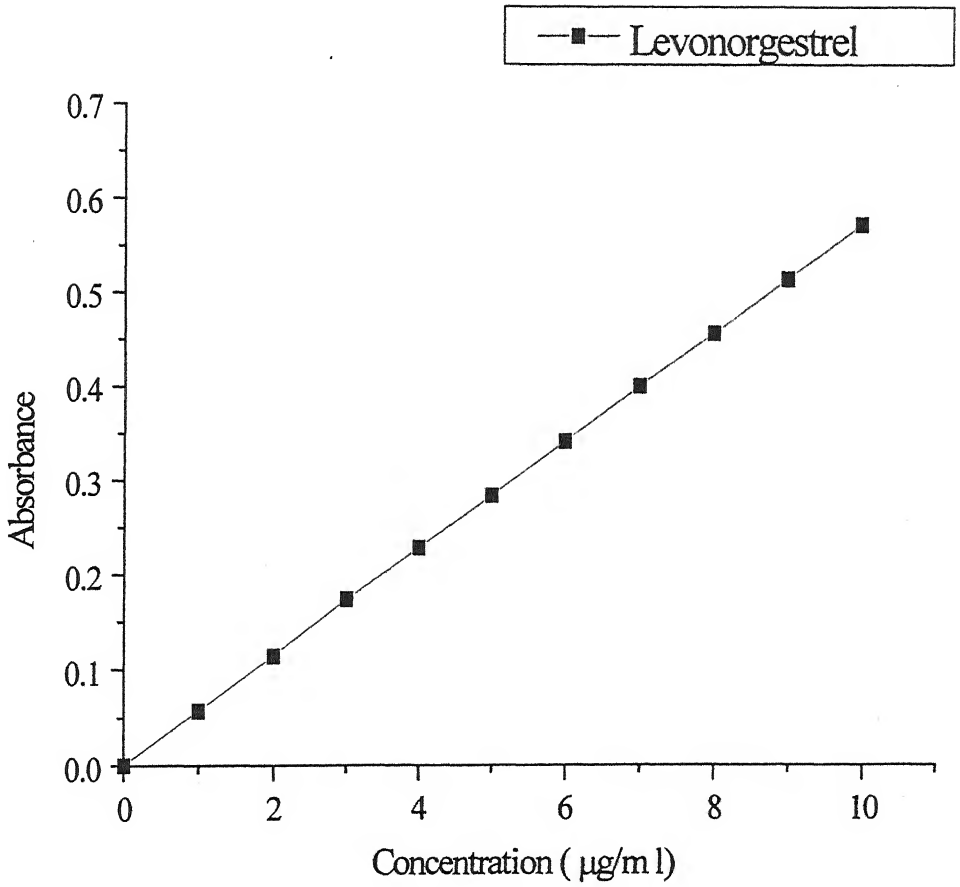


Figure 2.6: Standard Curve of Levonorgestrel in 40% PEG-200 at 247 nm

Where $A\lambda_1$ and $A\lambda_2$ are the measured absorbances at two wavelengths λ_1 and λ_2, the subscript 1 and 2 refer to the two different wavelengths. The wavelengths are selected to coincide with the absorption maxima of two solutes and should not overlap appreciably, so that substance 1 absorbs strongly at wavelength λ_1 and weekly at λ_2 and substance 2 absorbs strongly at λ_2 and weekly at λ_1. Now $A = \varepsilon cl$, where ε is the molar absorption coefficient at any particular wavelength, C is the concentration expressed in mol^{-1}, and l is the thickness (path length) of the absorbing solution expressed in 1 cm. If l is 1 cm then,

$$A\lambda_1 = \lambda_1\varepsilon_1c_1+\lambda_1\varepsilon_2c_2$$

$$A\lambda_2 = \lambda_2\varepsilon_1c_1+\lambda_2\varepsilon_2c_2$$

Solution of these simultaneous equations gives

$$C_1 = \frac{\lambda_1\varepsilon_2 A\lambda_1 - \lambda_1\varepsilon_2 A\lambda_2}{\lambda_1\varepsilon_1\lambda_2\varepsilon_2 - \lambda_1\varepsilon_2\lambda_2\varepsilon_1} \qquad C_2 = \frac{\lambda_1\varepsilon_1 A\lambda_2 - \lambda_2\varepsilon_1 A\lambda_1}{\lambda_1\varepsilon_1\lambda_2\varepsilon_2 - \lambda_1\varepsilon_2\lambda_2\varepsilon_1}$$

The value of molar absorption coefficient ε_1 and ε_2 can be deduced from measurements of the absorbances of pure solutions of substances 1 and 2. By measuring the absorbance of the mixture at wavelengths λ_1 and λ_2, the concentrations of the two components can be calculated. Same principle is applied in the case of multicomponent mode. Various combinations of ethinylestradiol and levonorgestrel; and estradiol plus levonorgestrel are commercially available. The literature survey revealed that HPLC and triple wavelength UV spectroscopy are the only methods reported for this combination. In the present study a simple UV spectrophotometric method using multicomponent mode was used for the determination of drugs in these combinations. The minimum concentration of drug to be analyzed should be 1 μg/ml.

General procedure for estimation of both drugs in combination:

Accurately weighed quantities 100 mg of ethinylestradiol, 100 mg of estradiol and 100 mg of levonorgestrel were transferred into three separate 100 ml of volumetric flasks. The weighed samples were dissolved in 40 ml of pure PEG 200, and volume made up to 100 ml with water. Then, 10 ml of ethinylestradiol, estradiol and levonorgestrel solutions were withdrawn into three 100 ml volumetric flask separately and again volume made upto 100 ml with the help 40% v/v PEG 200 solution to make the standard stock solution B of 100 μg/ml concentration of respective drugs.

Procedure:

From the stock solution B of each drug, 0.2, 0.4, 0.6, 0.8, 1.0, ... and 2.0 ml aliquots of solution of each drug (estradiol plus levonorgestrel in one series and ethinylestradiol plus levonorgestrel in another series of flasks) respectively were pipetted into a series of 10 ml volumetric flasks and volume made upto 10 ml with 40% v/v PEG 200. Thus solutions of both the drugs (ethinylestradiol + levonorgestrel and estradiol + levonorgestrel) containing mixed concentration in the range of 2 μg/ml to 20 μg/ml were obtained. These solutions were subjected to UV analysis using Shimadzu 1700 UV Spectrophotometer, Japan, through multicomponent mode by defining wave length, number of components of pure drug sample and scanning standard sample at 280 and 247 nm (for estradiol and levonorgestrel) and another combined sample solution at 281 and 247 nm (for ethinylestradiol and levonorgestrel). The observations are given in table 2.2.

Table 2.2: Absorbance data for standard curve of estradiol, ethinylestradiol, and levonorgestrel

S. No.	Standard Curve of estradiol at 280nm		Standard Curve of ethinylestradiol at 281 nm		Standard Curve of levonorgestrel at 247 nm	
	Concentration in μg/ml	Absorbance	Concentration in μg/ml	Absorbance	Concentration in μg/ml	Absorbance
1	1.0	0.050	1.0	0.076	1.0	0.057
2	2.0	0.115	2.0	0.151	2.0	0.114
3	3.0	0.178	3.0	0.225	3.0	0.174
4	4.0	0.243	4.0	0.301	4.0	0.229
5	5.0	0.325	5.0	0.375	5.0	0.285
6	6.0	0.374	6.0	0.451	6.0	0.343
7	7.0	0.437	7.0	0.525	7.0	0.402
8	8.0	0.503	8.0	0.601	8.0	0.458
9	9.0	0.569	9.0	0.676	9.0	0.516
10	10.0	0.636	10.0	0.752	10.0	0.574

Table 2.3: Derived statistical parameters related to standard curve of estradiol, ethinylestradiol and levonorgestrel in 40% v/v PEG 200 in distilled water

S. No.	Parameters	Value for 40% v/v PEG 200 in distilled water		
		Estradiol	Ethinylestradiol	Levonorgestrel
1	Regression coefficient	0.99958	0.99981	0.99999
2	Intercept value on Y axis	-0.0095	0.00027	-0.00018
3	Equation of line	Y=0.06426x-0.0095	Y=0.07509x+0.00027	Y=0.05735x-0.00018

Table 2.4: Amount of component drugs found in simultaneous determination of combined formulations

S. No.	Amount taken in aliquots	Amount found		Amount taken in aliquots	Amount found	
	Estradiol and Levonorgestrel	Conc. at 280 nm (μg/ml) Estradiol	Conc. at 247 nm (μg/ml) Levonor-gestrel	Ethinyl-estradiol and Levonorgestrel	Conc. at 281 nm (μg/ml) Ethinyl-estradiol	Conc. at 247 nm (μg/ml) Levonor-gestrel
1	2.0+2.0	2.3112	2.0132	2.0+2.0	2.0024	2.0641
2	4.0+4.0	4.0062	4.0082	4.0+4.0	4.1204	4.0421
3	6.0+6.0	6.0089	6.0123	6.0+6.0	6.0323	6.0093
4	8.0+8.0	8.023	7.9988	8.0+8.0	8.1224	7.9981
5	10.0+10.0	10.456	10.321	10.0+10.0	10.224	19.998
6	12.0+12.0	12.002	12.332	12.0+12.0	12.147	12.129
7	14.0+14.0	14.005	14.280	14.0+14.0	14.101	14.006
8	16.0+16.0	15.999	16.005	16.0+16.0	16.003	16.168
9	18.0+18.0	18.004	18.017	18.0+18.0	18.029	18.111
10	20.0+20.0	20.247	20.222	20.0+20.0	20.137	20.144

Determination of Interference of additives in the estimation of drugs:

Formulation additives used either in the preparation of proniosomal gel (surfactants) or used in the formulation of niosomes may interfere in the estimation of the drugs. Hence the interference checked using the maximum concentration of additives used in the formulations. Additives were dissolved to standard solution of respective drug prepared in methanol. In case of insoluble additives, the suspension after agitation was filtered through Whatman no.1 filter paper and filtrate collected. 5.0 ml volume of solution were appropriately diluted and were subjected to UV scanning as well as absorbance measurement as per procedures described earlier at respective wavelengths for each drug and the absorbances is reported in table 2.5.

Table 2.5: Absorption of drugs with additives at respective wavelength

S. No.	Additives/Drug	Estradiol (280nm)	Ethinylestradiol (281nm)	Levonorgestrel (247nm)
1	Pure drug solution without additives	0.698	0.755	0.547
2	Span-20	0.674	0.732	0.542
3	Span-40	0.712	0.744	0.532
4	Span-60	0.695	0.694	0.499
5	Span-80	0.677	0.741	0.540
6	Tween-20	0.694	0.750	0.544
7	Tween-20	0.684	0.742	0.546
8	Tween-20	0.691	0.733	0.561
9	Tween-20	0.683	0.756	0.549
10	Cholesterol	0.684	0.711	0.536
11	Dicetylphosphate	0.699	0.699	0.588
12	Egg lecithin	0.599	0.709	0.599
13	Soya lecithin	0.697	0.789	0.500

RESULT AND DISCUSSION

UV spectrophotometer method (USP, NF, 1995) was selected for the analysis of estradiol, ethinylestradiol, and levonorgestrel in combined formulations. The method was extended to 40% v/v PEG 200 solution and standard curves were plotted using PEG 200 solution as reference to avoid the deviation in analysis of drugs. Further 40% v/v PEG 200 solution in distilled water was used for release rate studies of these drugs to maintain good sink condition.

After scanning of estradiol, ethinylestradiol and levonorgestrel in 40% v/v PEG 200 in distilled water, between 200-350nm. The absorption maxima (λ_{max}) for these drugs were exhibited at 280, 281 and 247 nm respectively. The standard curves of these drugs were found to follow Beer-Lambert's law in the concentration range studied (1.0μg – 10.0 μg/ml). The regression coefficients were found to 0.99958, 0.99981 and 0.99999 respectively, which are the clear indication of excellent linearity.

For combined formulation of these drugs, two combination of standard solution were formed and multicomponent mode was followed for the analysis of drugs. The method was found suitable for determination of drug content in combined formulations. The result indicated that no interference was encountered with common excipients like span 40, span 20, tweens, 20, 40, 60, 80, lipid composition and alcohols.

This method of analysis is simple accurate, sensitive and reproducible when used for analysis of drugs in combined formulations. The method was found to be quite time saving, thus can be adopted for routine analysis work in the quality control laboratories.

The interference of formulation additives in the estimation of drug was checked. The absorbance of drug solution was recorded in the presence of maximum concentration of additives. As shown in the table 2.5, no or neglible influence on the absorbance of drug solutions was observed at 280, 281 and 247nm for estradiol, ethinylestradiol and levonorgestrel respectively.

Chapter-3

PREFORMULATION STUDY

Prior to starting the preformulation studies, the principle investigators should collect the information on the known properties of the compounds and proposed development schedule. The product development research is usually targeted for a specific therapeutic area and potency relative to competitive products as well as probable human dosage forms. The information on the proposed mode of the drug administration as well as literature on the formulation, bioavailability and pharmacokinetics of similar drug/ products often proves useful when deciding how to optimize the new formulation. The objective of preformulation is the quantitation of those physical and chemical properties that will assist in developing a stable, safe and effective formulation with the maximum bioavailability.

The major area of preformulation study are bulk characterization (crystallinity and polymorphism, hygroscopicity, fine particle characterization, bulk density, flow properties), solubility analysis (ionization constant, pH partition profile, common ion effect, thermal effects, solubilization, partition coefficient, dissolution), stability analysis (stability in toxicological formulations, solution stability, pH rate profile, solid state stability, bulk stability and compatibility)

EXPERIMENTAL

The sample of ethinylestradiol, and levonorgestrel were procured as gift sample from Famy care India, Vadodara and estradiol from Sun Pharma Pvt Ltd, Vadodara, identified and characterized as per the requirement of B.P. (1993).

The spans, tweens, dicetylphosphate, cholesterol, egg lecithin, soya lecithin were also identified and characterized as per the requirement of USP (1995) and BP (1993) respectively.

IR spectrum: The scanning was done using KBr dispersion pellet. About 1 mg of each of finely powdered sample of drugs was intimately mixed with approximately 100 mg of potassium bromide (spectroscopic grade) in a glass mortar. The mixture was compressed into transparent disks with special dies in a moisture free atmosphere and IR spectra were obtained on IR Spectrophotometer (Shimadzu, Japan). The scanning was done between 4000 to 400 cm^{-1}. The spectra so obtained were compared with reported in official compendia. The IR spectra of estradiol, ethinylestradiol, and levonorgestrel are shown in figure 3.1, 3.2 and 3.3. Characteristics peaks attributable to functional groups present in the molecule of each drug were assigned to establish the identity (Table-3.1).

Table 3.1: Functional groups and corresponding IR peaks of each drug

Estradiol		Ethinylestradiol		Levonorgestrel	
Peak cm^{-1}	**Groups**	**Peak cm^{-1}**	**Groups**	**Peak cm^{-1}**	**Groups**
3564	O-H stretching (v)	3409	Phenolic OH group, O-H stretching (v)	3347	O-H stretching (v)
3263	Aliphatic C-H stretching (v)	3290	C≡C-H acetylenic stretching (v)	3267	Acetylinic C-H stretching (v)
		3026	C-H stretching (v) alkene	2832-2933	Aliphatic C-H stretching (v)
2929 & 2857	C-H Aromatic bending (v)	2800-3000	C-H stretching (v) Aromatic	1651	Conjugated C=O stretching (v)
		1619	Stretching (v) five membered ring	1617	C=C stretching (v)
		1586	Stretching (v) six membered ring	1446	Methyl C-H asymmetric stretching (v)
1450	Asymmetric bending (v) CH_3 group	1497	C=C ring stretching (v)		
1450	C=C stretching (v) Aromatic ring	1445	Asymmetric bending (v) (due to CH_3 group)		
		1357	O-H in plain bending (v)	1363	Methyl C-H symmetric bending (v)
1127	C-O stretching (v) Alcohol	1156	C-O stretching (v) phenol	1066	Alcoholic C-O stretching (v)
1012-1030	Methyl C- H (in plain) bending (v)	1020-1060	C-H in plain bending	764-654	Acetylinic stretching (v)
648-810	Aromatic (out of plain) C-H bending	621-880	C-H out of plain bending Aromatic	427 & 1335	On plain starching (v)

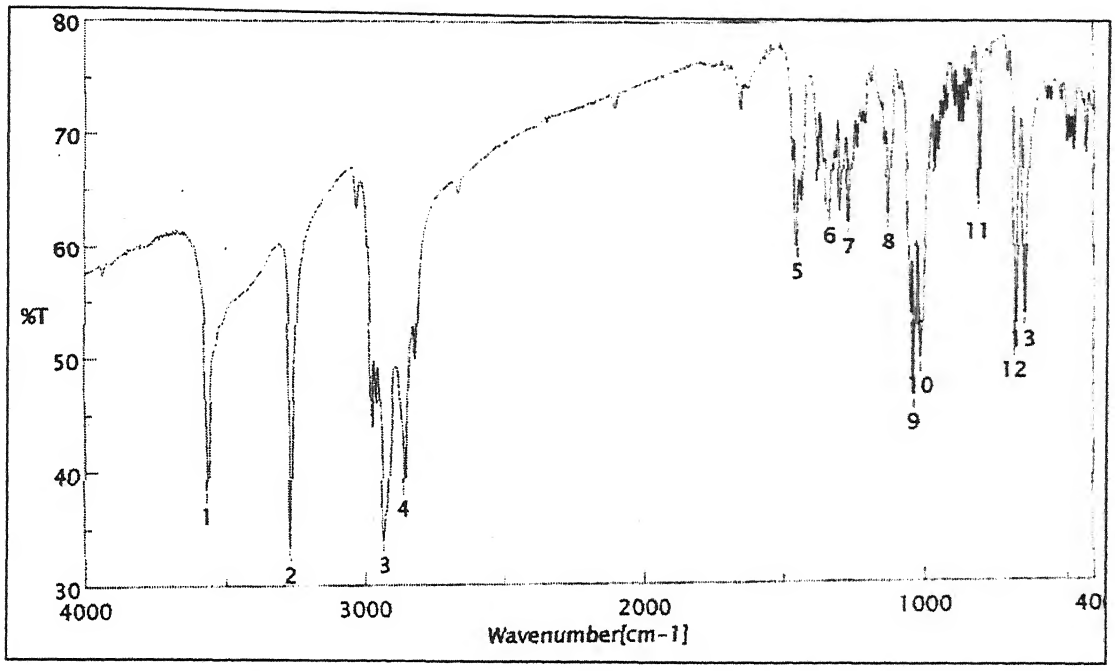


Figure 3.1: Infra-red spectrum of estradiol

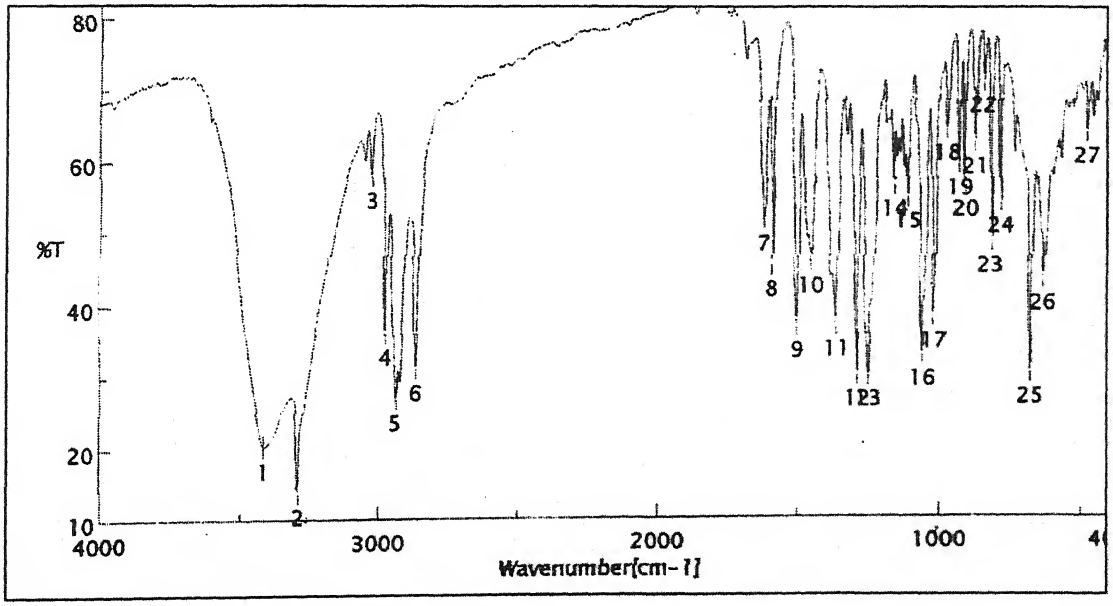


Figure 3.2: Infra-red spectrum of ethinylestradiol

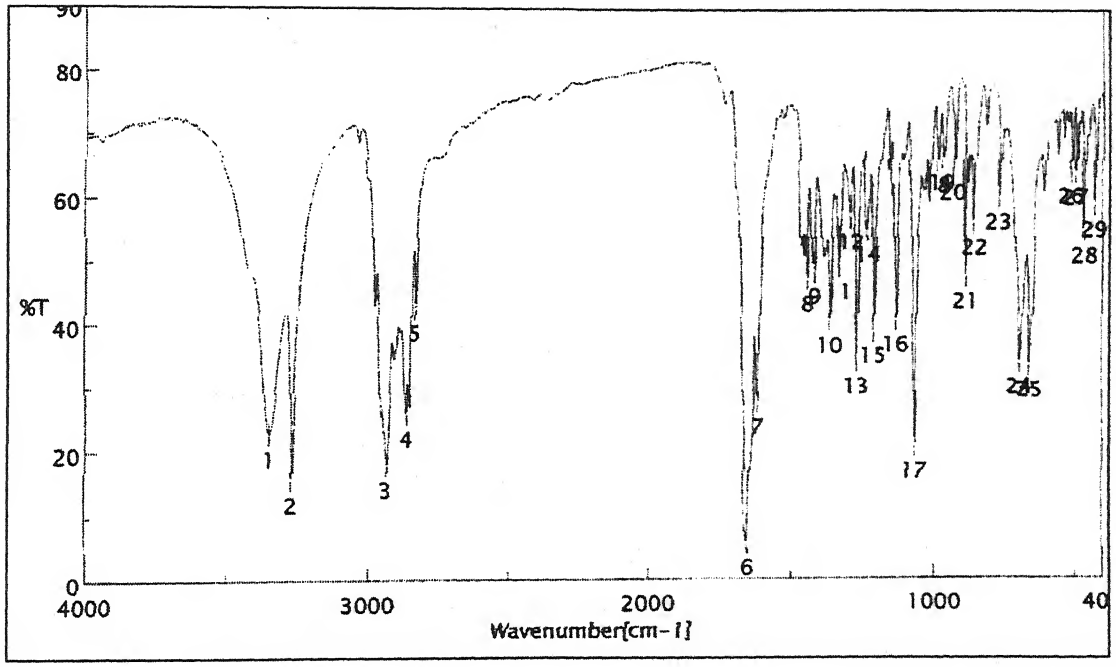


Figure-3.3: Infra-red spectrum of levonorgestrel

Solubility study of drugs: For equilibrium solubility determination the drug dispersed in solvent is agitated at a constant temperature. Samples of slurry are withdrawn as a function of time, clarified by centrifugation, and assayed to establish a plateau concentration.

The solubility study of drugs was performed in different solvents. A known quantity of drug (about 10mg) was suspended in a series of different solvents about (5.0 ml) in screw-capped test tubes. These test tubes were shaken in a mechanical wrist action shaker intermittently for 6 hr. at room temperature. After the incubation for equilibrium these test tube were observed weather the drug is soluble or not. A comparative solubility of drugs in different solvents are shown in table 3.2.

Partition coefficient: The measurement of drug's lipophilicity and indication of its ability to cross the lipoidal cell membrane is the oil/water partition coefficient in system such as octanol / water and chloroform /water. The partition coefficient is defined as the ratio of unionized drug distributed between the organic and aqueous phase at equilibrium. For a drug delivery system, the lipophilic/ hydrophilic balance has been shown to be contributing factor for rate and extent of drug absorption. Partition coefficient provides a means of characterizing the lipophilic/hydrophilic nature of drug.

The partition coefficient of drugs (estradiol, ethinylestradiol and levonorgestrel) was determined in two solvent systems e.g. n-octanol: water and n-octanol: aqueous 40% PEG 200. According to the method reported by Friend *et al.* (1990), accurately weighed quantity of (5.0 mg) drug taken in two stoppered test tubes containing 5 ml of n-octanol. After dissolving the drug in n-octanol, 5 ml water was added one tube and 5 ml of 40% PEG 200 was added to other glass stoppered test tubes. Then both the test tubes were kept to equilibrate by shaking in mechanical shaker for 6 hrs. All the tubes kept overnight for equilibrium. The contents were transferred into separating funnel and both phases were separated. After appropriate dilutions, the aqueous phase was analyzed for estradiol/ ethinylestradiol/ levonorgestrel against reagent blank solution using Simadzu-1700 UV spectrophotometer. The drug concentration in n-octanol phase was determined by subfracting the amount in aqueous phase from the total quantity of drug added to the tubes. The partition coefficient value "P" was calculated by the following equation.

$$P_{o/w} = (C_{organic} / C_{aqueous})$$

The results are reported in table 3.3.

Table 3.2: Solubility of Estradiol, Ethinylestradiol and Levonorgestrel in different solvents

S. No	Solvents	Comparative solubility in different solvents		
		Estradiol	Ethinylestradiol	Levonorgestrel
1	Water	---	---	---
2	Phosphate buffer (pH-7.4)	---	---	---
3	Absolute alcohol	++	++	++
4	Ethanol (95%)	++	++	++
5	Isopropyl alcohol	+++	+++	+++
6	Propanol	++	++	++
7	Butanol	++	++	++
8	Chloroform	+++	++	++
9	Acetonitrile	+++	+++	+++
10	Dichloromethane	+++	+++	+++
11	Methanol	++	++	++
12	Ether	+++	+++	+++
13	Acetone	+	+	+
14	Polyethylene glycol-200	+++	+++	+++
15	Polyethylene glycol 400	+++	+++	+++
16	Polyethylene glycol-600	+++	+++	+++
17	N-octanol	+++	+++	+++
18	Ethyl Acetate	++	++	++

--- = Insoluble, + = Sparingly soluble, ++ = Soluble, +++ = Very soluble

Table 3.3: Partition coefficient values of Estradiol, Ethinylestradiol & Levonorgestrel in n-octanol: water and n-octanol: 40%v/v PEG 200 aqueous solution

S. No.	Drug	Partition Coefficient Value	
		n-octanol:Water	n-octanol: 40%v/vPEG 200
1	**Estradiol**	2.062	1.022
2	**Ethinylestradiol**	1.074	0.900
3	**Levonorgestrel**	0.893	0.524

Table 3.4: Identification and characterization of estradiol

S. No.	Parameter	Observation
1	IR Spectrum	Figure 3.1
2	0.01% w/v solution in ethanol (95%) observed spectrophotometrically	Exhibits maxima at 280 nm
3	Heat 0.05 mg with 1.0 ml of 2.5% w/w solution of β-naphthol in sulphuric acid for 2 minutes at 100°C, cool and 1 ml of water;	An orange yellow colour is produced, which changed to red when the solution is heated for 90 sec. at 100°C
4	Melting point	178°C
5	Solubility	Practically insoluble in water, freely soluble in alcohol, ether and sparingly soluble in chloroform, soluble in 40% PEG 200, 400

Table 3.5: Identification and characterization of Ethinylestradiol

S. No.	Parameter	Observation
1	IR Spectrum	Figure 3.2
2	0.01% w/v solution in ethanol (95%) observed spectrophotometrically	Exhibits maxima at 281 nm
3	1.0 mg in 1 ml of sulphuric acid which exhibits a greenish fluorescence and when examined under UV light (365 nm). Add the solution to 10.0 ml water	Colour changes orange to violet
4	Melting point	184 °C
5	Solubility	Practically insoluble in water, freely soluble in alcohol, ether and sparingly soluble in chloroform, soluble in 40% PEG 200, 400

Table 3.6: Identification and characterization of Levonorgestrel

S. No.	Parameter	Observation
1	IR Spectrum	Figure 3.3
2	0.01% w/v solution in methanol observed spectrophotometrically	Exhibits maxima at 247 nm
3	0.2 mg drug + 0.3 ml of 0.5%, 1-3 dinitrobenzene + 0.1 ml of benzylkonium chloride + 2.0 ml 5 M Ammonia Test	Pink colour
4	Melting point	208°C
5	Solubility	Practically insoluble in water, sparingly soluble in chloroform, slightly soluble in acetone ethanol, ether, alcohol, ether and soluble in 40% PEG 200, 400

RESULT AND DISCUSSION:

The procured drug samples were identified by the various identification tests reported in I.P. and B.P. The absorption maxima of estradiol, ethinylestradiol, and levonorgestrel in methanol were obtained at 280, 281 and 247 nm respectively, which are concordant with the values reported in BP, 1993.

The identification of each drug sample was confirmed by comparing the I.R. spectrum of drug sample with that published in the standard texts/official compendia and characteristic peaks attributable to functional groups present in the molecule were assigned to establish the identity. The I.R. spectrum of estradiol, ethinylestradiol, and levonorgestrel was found to be in agreement to that reported in BP 1998 (Figure 3.1, 3.2 and 3.3).

The solubility determination of estradiol, ethinylestradiol and levonorgestrel was performed in common aqueous and organic solvents. Estradiol, ethinylestradiol, and levonorgestrel showed greater solubility in alcohol, ether, PEG-200, acetonitrile and they were found to be sparingly soluble in chloroform and insoluble in water. The partition coefficient of estradiol, ethinylestradiol, and levonorgestrel in n-octanol water system were found to be 2.062, 1.074 and 0.893 respectively, which are indicative of lipophilic nature of drugs. The lipophilic nature was found in following order estradiol > ethinylestradiol > levonorgestrel.

The lipophilic nature of drug favors the increased stability and decreased drug leakage from proniosomal gel system and hydrophilic viable epidermis may impose a significant barrier to the transdermal flux of estradiol, ethinylestradiol, and levonorgestrel. This property indicated the need of penetration enhancer in the transdermal formulation.

Chapter-4

FORMULATION OPTIMIZATION AND CHARACTERIZATION

Niosomes are unilamellar or multilamellar vesicles formed from synthetic non-ionic surfactants offering an alternative to liposomes as drug carriers. An increasing number of non-ionic surfactant has been found to form vesicles capable of entrapping hydrophilic and hydrophobic solutes.

It had been established that niosomes are unable to penetrate the skin; therefore, we have thought to investigate the co-delivery of non-ionic surfactants on the transdermal flux of estradiol, ethinylestradiol and levonorgestrel. In order to optimize the formulations, combination of spans and tweens either with or without unsaturated fatty acid additives, which are typically found in commercial supplied Egg lecithin and Soya lecithin were also tried. This system treated as proniosomal gel offers a versatile vesicle delivery concept with potential for delivery drugs via transdermal route (Vora *et al.*, 1998). The proposed system has some added advantages like greater stability, higher entrapment, greater membrane permeability and enhanced penetration (Kumhar *et al.*, 2003).

Various proniosomal formulations with different additives and different eight types of combination of drugs were devised, which are as follows:

a. Estradiol alone
b. Ethinylestradiol alone
c. Levonorgestrel alone
d. Estradiol alone with egg lecithin, soya lecithin, dicetylphosphate, cholesterol.
e. Ethinylestradiol with egg lecithin, soya lecithin, dicetylphosphate, cholesterol.
f. Levonorgestrel with egg lecithin, soya lecithin, dicetylphosphate, cholesterol.
g. Estradiol+ Levonorgestrel combined formulation
h. Ethinylestradiol + Levonorgestrel combined formulation

Total formulations were designed, the formulae of which are given in table 4 and 4a.

PREPARATION OF PRONIOSOMES:

Proniosomes were prepared by the method reported by Perrett *et al.*, (1991) with slight modification. The required quantities of isopropyl alcohol and drug were taken in a clean and dry wide mouthed glass bottles of 5.0 ml capacity. After warming, all the ingredients were mixed well with a glass rod, the open end of glass bottle was covered with a lid to prevent the loss of solvent from it and warmed over water bath at 60-70°C for about 5 minutes until the surfactant mixture was dissolved completely. Then aqueous

Table 4: Composition of proniosomal formulations using different ratios of tweens

S. No	Surfactant	Formulation code	Ratio of surfactant	Volume of Iso-propyl alcohol in ml	No. of drops of Water	Appearance
1	$T_{20}+T_{40}$	TT241	100+100	0.5	6	Yellowish viscous liquid
2	$T_{20}+T_{40}$	TT242	200+100	0.7	9	Yellowish viscous liquid
3	**$T_{20}+T_{40}$**	**TT243**	**300+100**	**1.0**	**9**	**Yellowish viscous liquid**
4	$T_{20}+T_{40}$	TT244	400+100	1.5	9	Yellowish viscous liquid
5	$T_{20}+T_{60}$	TT261	100+100	0.5	6	Yellowish translucent gel
6	$T_{20}+T_{60}$	TT262	200+100	0.7	9	Yellowish translucent gel
7	**$T_{20}+T_{60}$**	**TT263**	**300+100**	**1.0**	**9**	**Yellowish translucent gel**
8	$T_{20}+T_{60}$	TT264	400+100	1.5	9	Yellowish translucent gel
9	$T_{20}+T_{80}$	TT281	100+100	0.5	6	Yellowish translucent gel
10	$T_{20}+T_{80}$	TT282	200+100	0.7	9	Yellowish translucent gel
11	**$T_{20}+T_{80}$**	**TT283**	**300+100**	**1.0**	**9**	**Yellowish translucent gel**
12	$T_{20}+T_{80}$	TT284	400+100	1.5	9	Yellowish translucent gel
13	$T_{40}+T_{60}$	TT461	100+100	0.5	6	Yellowish translucent gel
14	$T_{40}+T_{60}$	TT462	200+100	0.7	9	Yellowish translucent gel
15	**$T_{40}+T_{60}$**	**TT463**	**300+100**	**1.0**	**9**	**Yellowish translucent gel**
16	$T_{40}+T_{60}$	TT464	400+100	1.5	9	Yellowish translucent gel
17	$T_{80}+T_{60}$	TT861	100+100	0.5	6	Viscous liquid solution
18	$T_{80}+T_{60}$	TT862	200+100	0.7	9	Viscous liquid solution
19	**$T_{80}+T_{60}$**	**TT863**	**300+100**	**1.0**	**9**	**Viscous liquid solution**
20	$T_{80}+T_{60}$	TT864	400+100	1.5	9	Viscous liquid solution
21	$T_{80}+T_{40}$	TT841	100+100	0.5	6	Yellowish viscous solution
22	$T_{80}+T_{40}$	TT842	200+100	0.7	9	Yellowish viscous solution
23	**$T_{80}+T_{40}$**	**TT843**	**300+100**	**1.0**	**9**	**Yellowish viscous solution**
24	$T_{80}+T_{40}$	TT844	400+100	1.5	9	Yellowish viscous solution

Cont'd.....

S. No	Surfactant	Formulation code	Ratio of surfactant	Volume of Iso-propyl alcohol in ml	No. of drops of Water	Appearance
25	$T_{20} + T_{40} + CHL$	TT241c	100+100+100	0.5	6	Yellowish translucent gel with cluster like structure of liquid crystal
26	$T_{20} + T_{40} + CHL$	TT242c	200+100+100	0.7	9	Yellowish translucent gel with cluster like structure of liquid crystal
27	**$T_{20} + T_{40} + CHL$**	**TT243c**	**300+100+100**	**1.0**	**9**	Yellowish translucent gel with cluster like structure of liquid crystal
28	$T_{20} + T_{40} + CHL$	TT244c	400+100+100	1.5	9	Yellowish translucent gel with cluster like structure of liquid crystal
29	$T_{40} + T_{60} + CHL$	TT461c	100+100+100	0.5	6	Creamish translucent gel with liquid crystal in irregular square shape
30	$T_{40} + T_{60} + CHL$	TT462c	200+100+100	0.7	9	Creamish translucent gel with liquid crystal in irregular square shape
31	**$T_{40} + T_{60} + CHL$**	**TT463c**	**300+100+100**	**1.0**	**9**	Creamish translucent gel with liquid crystal in irregular square shape
32	$T_{40} + T_{60} + CHL$	TT464c	400+100+100	1.5	9	Creamish translucent gel with liquid crystal in irregular square shape
33	$T_{80} + T_{60} + CHL$	TT861c	100+100+100	0.5	6	Whitish translucent gel with needle like liquid crystals
34	$T_{80} + T_{60} + CHL$	TT862c	200+100+100	0.7	9	Whitish translucent gel with needle like liquid crystals
35	**$T_{80} + T_{60} + CHL$**	**TT863c**	**300+100+100**	**1.0**	**9**	Whitish translucent gel with needle like liquid crystals
36	$T_{80} + T_{60} + CHL$	TT864c	400+100+100	1.5	9	Whitish translucent gel with needle like liquid crystals
37	$T_{80} + T_{40} + CHL$	TT841c	100+100+100	0.5	6	Creamish translucent gel with square layered structure
38	$T_{80} + T_{40} + CHL$	TT842c	200+100+100	0.7	9	Creamish translucent gel with square layered structure
39	**$T_{80} + T_{40} + CHL$**	**TT843c**	**300+100+100**	**1.0**	**9**	Creamish translucent gel with square layered structure
40	$T_{80} + T_{40} + CHL$	TT844c	400+100+100	1.5	9	Creamish translucent gel with square layered structure

Table 4a: Composition of proniosomal formulations using different ratios of Spans & tweens

S. No	Surfactant	Formulation code	Ratio of surfactant (mg)	Volume of Iso- propyl alcohol (ml)	No. of drops of Water	Appearance
41	$S_{20} + T_{20}$	ST221	100+100	0.5	6	Brownish optical anisotropic gel
42	$S_{20} + T_{20}$	ST222	200+100	0.7	9	Brownish optical anisotropic gel
43	**$S_{20} + T_{20}$**	**ST223**	**300+100**	**1.0**	**9**	Brownish optical anisotropic gel
44	$S_{40} + T_{20}$	ST224	400+100	1.5	9	Brownish optical anisotropic gel
45	$S_{40} + T_{20(EEE)}$	ST221	100+100	0.5	6	Brownish translucent semisolid gel
46	$S_{40} + T_{20(EEE)}$	ST222	200+100	0.7	9	Brownish translucent semisolid gel
47	**$S_{40} + T_{20(EEE)}$**	**ST223**	**300+100**	**1.0**	**9**	Brownish translucent semisolid gel
48	$S_{40} + T_{20(EEE)}$	ST224	400+100	1.5	9	Brownish translucent semisolid gel
49	$S_{60} + T_{20}$	ST621	100+100	0.5	6	Whitish translucent gel
50	$S_{60} + T_{20}$	ST622	200+100	0.7	9	Whitish translucent gel
51	**$S_{60} + T_{20}$**	**ST623**	**300+100**	**1.0**	**9**	Whitish translucent gel
52	$S_{60} + T_{20}$	ST624	400+100	1.5	9	Whitish translucent gel
53	$S_{80} + T_{20}$	ST821	100+100	0.5	6	Yellowish translucent gel
54	$S_{80} + T_{20}$	ST822	200+100	0.7	9	Yellowish translucent gel
55	**$S_{80} + T_{20}$**	**ST823**	**300+100**	**1.0**	**9**	Yellowish translucent gel
56	$S_{80} + T_{20}$	ST824	400+100	1.5	9	Yellowish translucent gel
57	$S_{40} + T_{20(EEE)}$	ST421	100+100	0.5	6	Creamish brown Semisolid gel
58	$S_{40} + T_{20(EEE)}$	ST422	200+100	0.7	9	Creamish brown Semisolid gel
59	**$S_{40} + T_{20(EEE)}$**	**ST423**	**300+100**	**1.0**	**9**	Creamish brown translucent gel
60	$S_{40} + T_{20(EEE)}$	ST424	400+100	1.5	9	Creamish brown translucent gel
61	$S_{40} + T_{40}$	ST441	100+100	0.5	6	Creamish semisolid gel
62	$S_{40} + T_{40}$	ST442	200+100	0.7	9	Creamish semisolid gel
63	**$S_{40} + T_{40}$**	**ST443**	**300+100**	**1.0**	**9**	Creamish Translucent gel
64	$S_{40} + T_{40}$	ST444	400+100	1.5	9	Creamish Translucent gel
65	$S_{40} + T_{60}$	ST461	100+100	0.5	6	Creamish semisolid gel
66	$S_{40} + T_{60}$	ST462	200+100	0.7	9	Creamish semisolid gel
67	**$S_{40} + T_{60}$**	**ST463**	**300+100**	**1.0**	**9**	Creamish Translucent gel
68	$S_{40} + T_{60}$	ST464	400+100	1.5	9	Creamish Translucent gel
69	$S_{40} + T_{80}$	ST481	100+100	0.5	6	Brownish white semisolid gel
70	$S_{40} + T_{80}$	ST482	200+100	0.7	9	Brownish white semisolid gel

Cont'd.....

S. No	Surfactant	Formulation code	Ratio of surfactant (mg)	Volume of Iso- propyl alcohol (ml)	No. of drops of Water	Appearance
71	**$S_{40} + T_{80}$**	**ST483**	**300+100**	**1.0**	**9**	Brownish white translucent gel
72	$S_{40} + T_{80}$	ST484	400+100	1.5	9	Brownish white translucent gel
73	$S_{60} + T_{20}$	ST621	100+100	0.5	6	Whitish semisolid gel
74	$S_{60} + T_{20}$	ST622	200+100	0.7	9	Whitish semisolid gel
75	**$S_{60} + T_{20}$**	**ST623**	**300+100**	**1.0**	**9**	Whitish translucent gel
76	$S_{60} + T_{20}$	ST624	400+100	1.5	9	Whitish translucent gel
77	$S_{60} + T_{40}$	ST641	100+100	0.5	6	Whitish semisolid gel
78	$S_{60} + T_{40}$	ST642	200+100	0.7	9	Whitish semisolid gel
79	**$S_{60} + T_{40}$**	**ST643**	**300+100**	**1.0**	**9**	Whitish translucent gel
80	$S_{60} + T_{40}$	ST644	400+100	1.5	9	Whitish translucent gel
81	$S_{60} + T_{60}$	ST661	100+100	0.5	6	Creamish white gel
82	$S_{60} + T_{60}$	ST662	200+100	0.7	9	Creamish white gel
83	**$S_{60} + T_{60}$**	**ST663**	**300+100**	**1.0**	**9**	Creamish white translucent gel
84	$S_{60} + T_{60}$	ST664	400+100	1.5	9	Creamish white translucent gel
85	$S_{60} + T_{80}$	ST681	100+100	0.5	6	Whitish semisolid gel
86	$S_{60} + T_{80}$	ST682	200+100	0.7	9	Whitish semisolid gel
87	**$S_{60} + T_{80}$**	**ST683**	**300+100**	**1.0**	**9**	Whitish semisolid translucent gel
88	$S_{60} + T_{80}$	ST684	400+100	1.5	9	Whitish semisolid translucent gel
89	$S_{80} + T_{20}$	ST821	100+100	0.5	6	Creamish translucent gel
90	$S_{80} + T_{20}$	ST822	200+100	0.7	9	Creamish translucent gel
91	**$S_{80} + T_{20}$**	**ST823**	**300+100**	**1.0**	**9**	Creamish translucent gel
92	$S_{80} + T_{20}$	ST824	400+100	1.5	9	Creamish translucent gel
93	$S_{80} + T_{40}$	ST841	100+100	0.5	6	Yellowish viscous liquid
94	$S_{80} + T_{40}$	ST842	200+100	0.7	9	Yellowish viscous liquid
95	**$S_{80} + T_{40}$**	**ST843**	**300+100**	**1.0**	**9**	Yellowish viscous liquid
96	$S_{80} + T_{40}$	ST844	400+100	1.5	9	Yellowish viscous liquid
97	$S_{80} + T_{60}$	ST861	100+100	0.5	6	Creamish translucent gel
98	$S_{80} + T_{60}$	ST862	200+100	0.7	9	Creamish translucent gel
99	**$S_{80} + T_{60}$**	**ST863**	**300+100**	**1.0**	**9**	Yellowish viscous liquid
100	$S_{80} + T_{60}$	ST864	400+100	1.5	9	Yellowish viscous liquid

Cont'd.....

S. No	Surfactant	Formulation code	Ratio of surfactant	Volume of Iso- propyl alcohol in ml	No. of drops of Water	Appearance
101	$S_{80} + T_{80}$	ST881	100+100	0.5	6	Yellowish optical anisotropic translucent gel
102	$S_{80} + T_{80}$	ST882	200+100	0.7	9	Yellowish optical anisotropic translucent gel
103	**$S_{80} + T_{80}$**	**ST883**	**300+100**	**1.0**	**9**	Yellowish optical anisotropic translucent gel
104	$S_{80} + T_{80}$	ST884	400+100	1.5	9	Yellowish viscous gel
105	$S_{40} + S_{20}$	SS241	100+100	0.5	6	Brownish optical anisotropic gel
106	$S_{40} + S_{20}$	SS242	200+100	0.7	9	Brownish optical anisotropic gel
107	$S_{40} + S_{20}$	SS243	**300+100**	**1.0**	**9**	Brownish optical anisotropic gel
108	$S_{40} + S_{20}$	SS244	400+100	1.5	9	Brownish optical anisotropic gel

Cont'd.....

Proniosomal formulations selected for characterization

S. No	Surfactant	Formulation code	Ratio of surfactant	Volume of Iso- propyl alcohol in ml	No. of drops of Water	Appearance
109	$S_{40} + T_{20(IPA)}$	*ST423*	300+100	1.0	9	Brownish (optical anisotropic) translucent gel
110	$S_{40} + T_{20(AA)}$	*ST423*	300+100	1.0	9	Brownish (optical anisotropic) translucent gel
111	$S_{40} + T_{20(P)}$	*ST423*	300+100	1.0	9	Brownish (optical anisotropic) translucent gel
112	$S_{40} + T_{20(B)}$	*ST423*	300+100	1.0	9	Brownish (optical anisotropic) translucent gel
113	$S_{40} + T_{20(17\beta)}$	ST423	300+100	1.0	9	Brownish (optical anisotropic) translucent gel
114	$S_{40} + T_{20(17\beta)}$	ST423	300+100	1.0	9	Brownish (optical anisotropic) translucent gel
115	$S_{40} + T_{20(17\beta)}$	ST423	300+100	1.0	9	Brownish (optical anisotropic) translucent gel
116	$S_{40} + T_{20(17\beta)}$	ST423	300+100	1.0	9	Brownish (optical anisotropic) translucent gel
117	$S_{40} + T_{20(LN)}$	ST423	300+100	1.0	9	Brownish (optical anisotropic) translucent gel
118	$S_{40} + T_{20(LN)}$	ST423	300+100	1.0	9	Brownish (optical anisotropic) translucent gel
119	$S_{40} + T_{20(LN)}$	ST423	300+100	1.0	9	Brownish (optical anisotropic) translucent gel
120	$S_{40} + T_{20(LN)}$	ST423	300+100	1.0	9	Brownish (optical anisotropic) translucent gel
121	$S_{40} + T_{20(EL)}$	ST423 (EL)	300+100+100	1.0	9	Brownish (optical anisotropic) translucent gel
122	$S_{40} + T_{20(SL)}$	ST423 (SL)	300+100+100	1.0	9	Brownish (optical anisotropic) translucent gel
123	$S_{40} + T_{20(DCP)}$	ST423 (DCP)	300+100+100	1.0	9	Brownish (optical anisotropic) translucent gel
124	$S_{40} + T_{20(CHL)}$	ST423 (CHL)	300+100+100	1.0	9	Brownish (optical anisotropic) translucent gel
125	$S_{40} + S_{20}$ (17β+LN)	S1	300+100	1.0	9	Brownish (optical anisotropic) translucent gel
126	$S_{40} + T_{20}$ (17β+LN)	T1	300+100	1.0	9	Brownish (optical anisotropic) translucent gel
127	$S_{40} + S_{20}$ (EEE+LN)	S1	300+100	1.0	9	Brownish (optical anisotropic) translucent gel
128	$S_{40} + T_{20}$ (EEE+LN)	T1	300+100	1.0	9	Brownish (optical anisotropic) translucent gel

EEE – Ethinylestradiol
17β - Estradiol
LN - Levonorgestrel

IPA – Isopropyl alcohol
AA – Absolute alcohol
P – Propanol
B – Butanol

EL - Egg lecithin
SL - Soya lecithin
DCP – Dicetyl phosphate
CHL - Cholesterol

phase containing (0.1% glycerol solution) was added and warmed on water bath till clear solution was formed which was converted into proniosomal gel on cooling. The gel so obtained was preserved in same glass bottle in dark for characterization. Similarly other proniosomal formulations were prepared, the compositions of which are given in table 4 and 4a.

CHARACTERIZATION OF PRONIOSOMES:

The prepared proniosomal gels bearing estradiol, ethinylestradiol and levonorgestrel drugs were characterized for the following parameters:

Shape of Vesicles:

A thin layer of proniosomal gel was spread on a concave or deep well cavity slide and covered with glass cover slip. The slide was observed under polarizing microscope with or without polarizing light to see the optical anisotropic crystals; a drop of water was added to the slide through the cover slip into the cavity slide while under optical microscope (Nikon HFX-OX; Labophot microscope) and again observed for the formation of vesicles. Photomicrographs were taken at suitable magnification before and after addition of water to the formulation (S1 and T1). This evaluation provided help in optimizing the system (Figure 4.1-4.3 and 4.4-4.6).

On the basis of microscopic evaluation of proniosomal formulations consistency, appearance, and rapidity of formation of vesicles on addition of water, two formulations SS42 (S1) and ST42 (T1) were chosen for drug loading and further studies.

Drug Crystallization in Proniosomal Gel:

In the optimized formulation prepared with combination of spans, spans and tweens, the varying amount of drugs were incorporated in order to see that what maximum of quantity of drug(s) could be incorporated without crystallization in the proniosomal formulations.

The formulation containing vary increasing amount of drug were examined under optical microscope at 1000X magnification for crystal appearance, if any. The observations are reported in table 4.1.

Size Distribution Study:

Size distribution study was performed in same manner of spontaneity formation of niosomes including hydration with agitation and hydration with sonication.

The proniosomal gel (100 mg) was hydrated in a small glass bottle using 10 ml saline solution (0.154 M, NaCl) with occasional shaking for 10 minutes. The dispersion

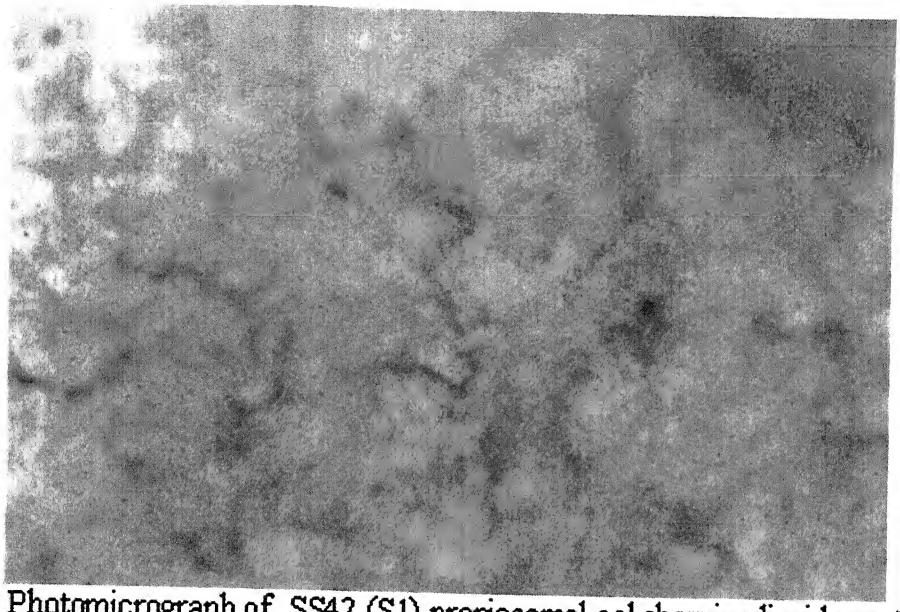


Figure-4.1: Photomicrograph of SS42 (S1) proniosomal gel showing liquid crystals in optical Anisotropic stage

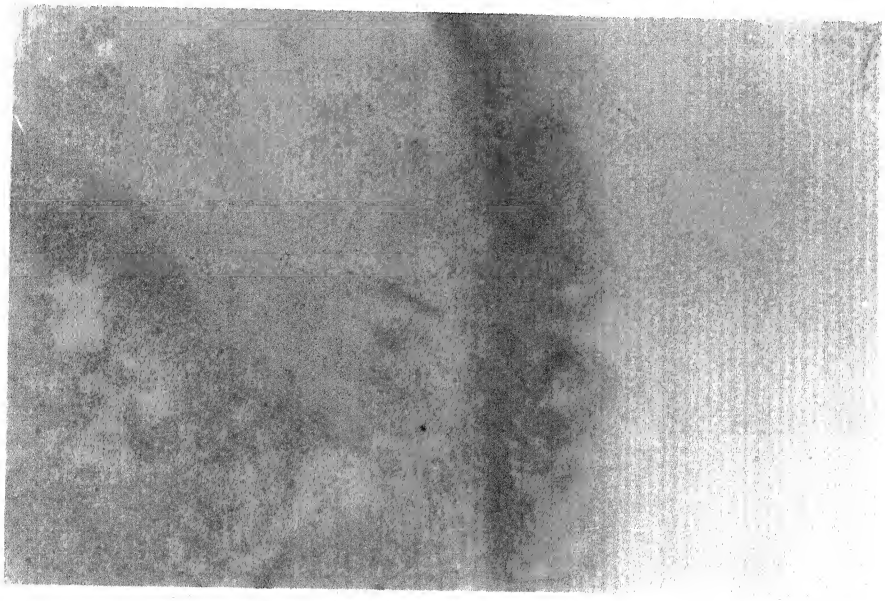


Figure-4.2: Photomicrograph of SS42 (S1) proniosomal gel showing bud like structure after hydration with water

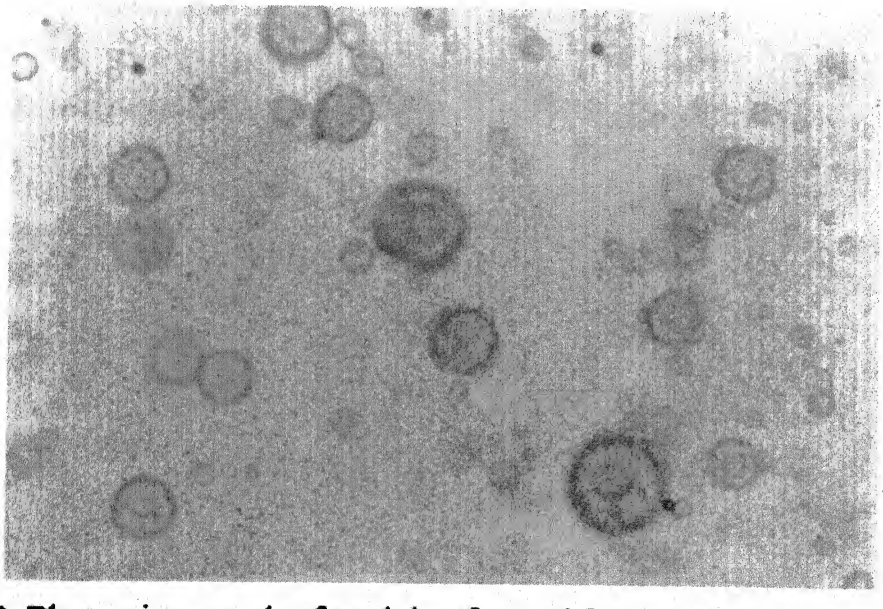


Figure-4.3: Photomicrograph of vesicles formed from SS42 (S1) proniosomal gel

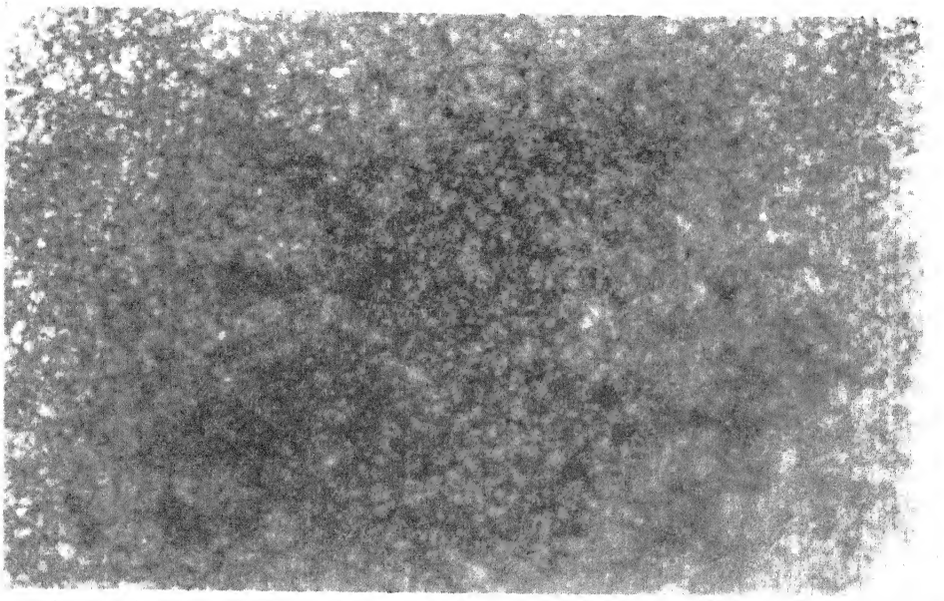


Figure-4.4: Photomicrograph of ST42 (T1) proniosomal gel showing liquid crystals in optical Anisotropic stage

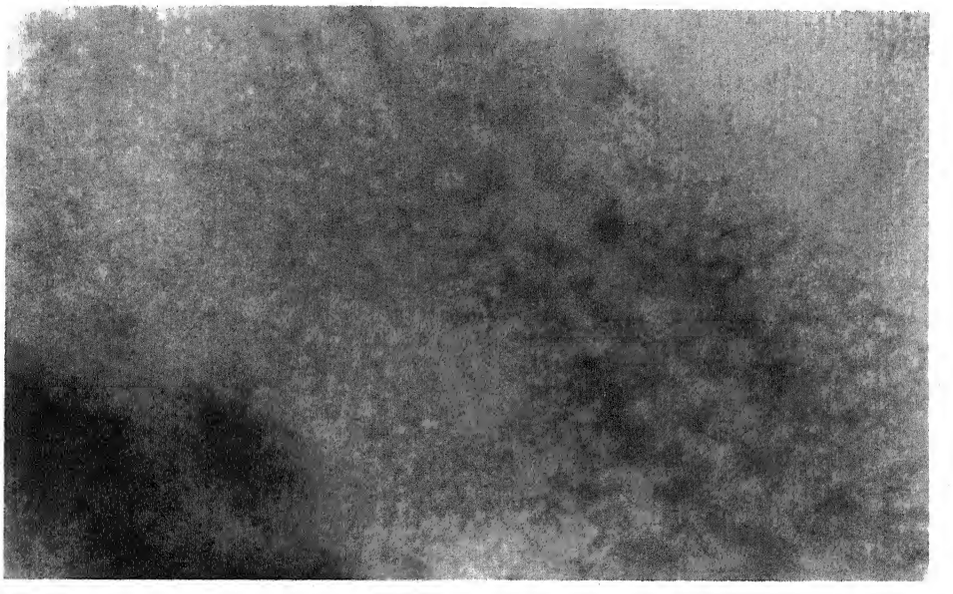


Figure-4.5: Photomicrograph of ST42 (T1) proniosomal gel showing bud like structure after hydration with water

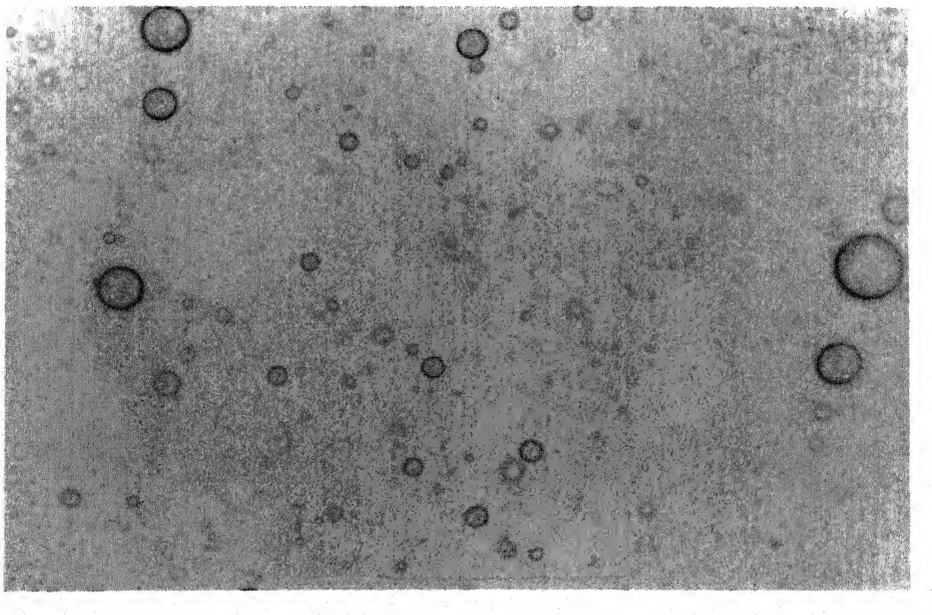


Figure-4.6: Photomicrograph of vesicles formed from ST42 (T1) proniosomal gel

was observed under optical microscope at 100x magnification. Size and size distribution of 200-300 vesicles was noted using calibrated ocular micrometer fitted in a Simple microscope (Elico Instruments, Hyderabad). Similarly sizes were noted for niosomes (200-300) formed spontaneously from other proniosomal gel, after hydration without and with agitation in a cavity slide. The observation are reported in table 4.2

Table 4.1: Observations for drug crystal appearance in optimized proniosomal formulations

Name	Amount of drug (mg)				
	1.0 mg	1.5 mg	2.0 mg	2.5 mg	3.0 mg
SS2411	CNA	CNA	CNA	CA	CA
SS2421	CNA	CNA	CNA	CNA	CA
SS2431	CNA	CNA	CNA	CNA	CA
SS2441	CNA	CNA	CNA	CNA	CA
ST22	CNA	CNA	CNA	CNA	CA
ST24	CNA	CNA	CNA	CNA	CAN
ST26	CNA	CNA	CNA	CNA	CAN
ST28	CNA	CNA	CNA	CNA	CAN
ST42	CNA	CNA	CNA	CA	CNA
ST44	CNA	CNA	CNA	CNA	CNA
ST46	CNA	CNA	CNA	CNA	CNA
ST48	CNA	CNA	CNA	CNA	CNA
ST62	CNA	CNA	CNA	CA	CA
ST64	CNA	CNA	CNA	CNA	CA
ST66	CNA	CNA	CNA	CNA	CA
ST68	CNA	CNA	CNA	CNA	CA
ST82	CNA	CNA	CNA	CNA	CA
ST84	CNA	CNA	CNA	CNA	CA
ST86	CNA	CNA	CNA	CNA	CA
ST88	CNA	CNA	CNA	CNA	CNA
ST42EL	CNA	CNA	CNA	CNA	CNA
ST42SL	CNA	CNA	CNA	CNA	CNA
ST42DCP	CNA	CNA	CNA	CNA	CNA
ST42CHL	CNA	CNA	CNA	CNA	CNA

* CA- Crystals appeared ** CNA- Crystals not appeared

Spontaneity of Niosome Formation:

This study was performed by Payne *et al.* (1986) for vesicle formation from proniosomal systems; spontaneity can be defined, as the number of niosomes formed after hydration of proniosomes for 15-20 minutes.

Approximately 10 or 20 mg of proniosomal gel was transferred to the bottom of clean-stoppered glass bottle and spread uniformly around the wall of the glass bottle with the help of a glass rod. At room temperature 2 ml of phosphate saline (0.154 M NaCl) was added carefully along the walls of the glass bottle and it was left in test tube stand.

After 20 minutes, a drop of this saline solution was withdrawn and placed on Neubaures chamber (Fein optic, Germany) to count the number of vesicles. The numbers of niosomes eluted from proniosomes were counted. The observations are recorded in table 4.2.

Drug Entrapment Efficiency:

For determining the drug entrapment efficiency of the vesicles, approximately 100 mg of proniosomal gel containing ethinylestradiol, estradiol & levonorgestrel was dispersed into the phosphate buffer saline (10 ml) and warmed little bit for the formation of niosomes. Free drug was separated from the niosomes entrapped the drugs. A small aliquot (5.0 ml) of niosomes was centrifuged. A stiff floating fraction-containing niosomes formed at the top of the tube, and a clear niosomes free solvent fraction. The clear fraction was used for the determination of the free drug. A small aliquot of the clear dispersion fraction was diluted upto 5.0 ml with 40% PEG 200 and this solution used to obtain the absorbance. The drug concentration in niosomes fraction was determined in similar manner, diluting a 0.2 ml of aliquot with triton-X 100 to disrupt the vesicle and then diluted with 5.0 ml of 40% PEG 200. The resulting solution was filtered and analyzed for drug content at 281, 280 and 247 nm respectively against the blank solution of 40% PEG 200 using Shimadzu-1700 UV spectrophotometer. The entrapment efficiency of the drugs was defined as the ratio of the mass niosomes associated drug to the total mass of drug. The observation are shown in table 4.2.

Table 4.2: Vesicle size of different formulations with drug entrapment efficiency of ethinylestradiol, estradiol and levonorgestrel.

S. No.	Formulation code	Vesicle size with hydration	Poly-dispersity Index	Vesicle size with agitation	Poly-dispersity Index	%Drug Entrapment
1	$SS4211_{(EEE)}$	8.84±0.11	0.015	1.60 ± 0.15	0.093	94.16±5.12
2	$SS4221_{(EEE)}$	8.99±0.19	0.021	1.40 ± 0.11	0.078	92.36±5.34
3	$SS4231_{(EEE)}$	10.11±0.15	0.014	1.67 ± 0.09	0.053	91.11±4.58
4	$SS4241_{(EEE)}$	15.44±0.27	0.017	1.78 ± 0.14	0.078	90.80±2.96
5	$ST22_{(EEE)}$	3.16±0.07	0.022	1.38 ± 0.02	0.014	78.12±1.18
6	$ST24_{(EEE)}$	3.82±0.05	0.013	1.42 ± 0.07	0.049	69.12±1.14
7	$ST26_{(EEE)}$	3.15±0.04	0.012	1.52 ± 0.08	0.052	67.42±3.86
8	$ST28_{(EEE)}$	3.01±0.12	0.039	1.29 ± 0.11	0.085	70.17±3.34
9	$ST42_{(EEE)}$	5.53±0.13	0.023	1.83 ± 0.01	0.005	90.12±5.63
10	$ST44_{(EEE)}$	5.89±0.09	0.015	1.63 ± 0.03	0.018	89.99±2.74
11	$ST46_{(EEE)}$	6.63±0.08	0.012	1.52 ± 0.06	0.039	88.86±6.22
12	$ST48_{(EEE)}$	5.44±0.07	0.012	1.54 ± 0.08	0.051	89.63±3.43
13	$ST62_{(EEE)}$	6.84±0.11	0.016	2.95 ± 0.13	0.044	89.88±6.63
14	$ST64_{(EEE)}$	6.02±0.14	0.023	2.92 ± 0.17	0.041	84.12±5.58
15	$ST66_{(EEE)}$	6.44±0.18	0.027	2.89 ± 0.11	0.038	80.38±7.12
16	$ST68_{(EEE)}$	6.77±0.09	0.013	2.90 ± 0.12	0.041	82.88±7.72
17	$ST82_{(EEE)}$	4.12±0.01	0.002	1.73 ± 0.09	0.052	70.17±3.33
18	$ST84_{(EEE)}$	4.85±0.11	0.022	2.29 ± 0.08	0.034	69.99±1.18
19	$ST86_{(EEE)}$	4.95±0.21	0.042	1.52 ± 0.04	0.026	65.84±2.24
20	$ST88_{(EEE)}$	5.22±0.02	0.003	1.52 ± 0.05	0.032	69.18±6.64
21	$ST42_{(AA)}$	5.88 ± 0.06	0.004	1.92 ± 0.07	0.024	87.66±3.57
22	$ST42_{(P)}$	6.12 ± 0.11	0.022	2.02 ± 0.14	0.033	89.62±8.21
23	$ST42_{(B)}$	6.88 ± 0.09	0.014	2.46 ± 0.23	0.034	89.94±4.44
24	$ST42_{EL(EEE)}$	7.74±0.11	0.014	3.33 ± 0.11	0.033	93.66±4.42
25	$ST42_{SL(EEE)}$	7.99±0.14	0.017	3.84 ± 0.17	0.044	91.34±4.77
26	$ST42_{DCP(EEE)}$	6.98±0.15	0.021	3.99 ± 0.19	0.047	90.14±3.98
27	$ST42_{CHL(EEE)}$	4.33±0.18	0.041	2.11 ± 0.20	0.141	89.82±2.41
28	$ST42_{(17\beta)}$	6.11±0.12	0.019	1.99 ± 0.12	0.012	94.22±6.60
29	$ST44_{(17\beta)}$	5.77±0.19	0.016	1.92 ± 0.23	0.022	93.13±3.34
30	$ST46_{(17\beta)}$	7.23±0.17	0.014	1.84 ± 0.16	0.029	92.96±7.22
31	$ST48_{(17\beta)}$	6.01±0.18	0.032	1.74 ± 0.08	0.061	91.77±6.41
32	$ST42_{(LN)}$	6.22±0.14	0.021	2.04 ± 0.14	0.022	93.66±7.71
33	$ST44_{(LN)}$	5.98±0.11	0.017	1.89 ± 0.25	0.032	92.13±4.42
34	$ST46_{(LN)}$	6.97±0.16	0.016	1.94 ± 0.18	0.033	91.68±2.78
35	$ST48_{(LN)}$	5.99±0.19	0.034	1.83 ± 0.17	0.051	91.34±5.41

Drug Release /Permeation Profile From Proniosomal Formulations:

Preparation of cellophane membrane:

Cellophane membrane generally contains 25% glycerol, 0.1% sulphur and some fatty material. It was prepared by subjecting to digestion with series of solution followed by washings. Placing them in distilled water and heating it for about an hour on boiling water bath removed the membrane glycerol. It was then transferred to fresh water and again the process was repeated. After this the membrane was kept in alcohol for one day and then it was washed several times with water. Lastly the membrane polysulphide was removed by treating the membrane with 0.3% w/v solution of sodium sulphide at 80°C for one minutes, washed with hot water (60°C) for 2 min followed by acidification with a 0.2% v/v solution of sulphuric acid, Finally it was rinsed with hot water to remove the acid.

Drug diffusion studies through cellophane membrane:

A locally fabricated and well-calibrated Keshary-chein (Jyoti Scientific, GWL) type permeation system was used in the cellophane permeation studies of estradiol, levonorgestrel, and ethinylestradiol. Freshly treated cellophane membrane was mounted between the half-cell of Keshary-chein permeation cell. The donor half which faced the upper surface patch containing proniosomal formulation with 10 mg drug was adhered to the outer side of the cellophane membrane, while receptor half cell, which faced the lower side, had a solution containing 40% v/v PEG 200 to maintain the sink condition required. The receptor compartment was surrounded by a water jacket for maintaining the temperature 37±1°C for 24 hrs, using a thermostatically controlled hot plate cum magnetic stirrer (Jyoti Scientific, GWL). The top of the donor compartment was open for air circulation. The capacity of receptor compartment was 10 ml. The area of donor compartment was 1.13 cm^2.

At predetermined time intervals (0.75 hr), 5.0 ml samples were taken from receptor solution, which was refilled with same volume of 40% v/v PEG 200 saline solution. Samples taken were analyzed spectrophotometrically for estradiol, ethinylestradiol, and levonorgestrel formulation in a UV 1700 (Shimadzu, Japan) against respective blank and amount of drug in 40% v/v PEG 200 saline solution and samples of combined formulation was analyzed by multi-component mode in same manner. The observations are recorded in table 4.3-4.13.

Preparation of Female Rat Skin and Drug Permeation Study of Drugs from Proniosomal Formulation Through Rat Skin:

The hairs of female rat weighing (Sprange Dawley strain) 180-200gm were first picked away with scissors and then depilatory (Ann-French) was applied for 20 min, which was then wiped off with cotton. After cleaning the skin with water, the animal was returned to cage. Next day the animal was sacrificed by inhalation of chloroform. An incision was made on the flank of the animal and skin was separated from the under lying connective tissue using scalpel. The fat still remaining on the skin was trimmed away by using saving razor blade. The prepared skin was then washed with saline (0.154M NaCl) and stored at 0-4°C after applying gentamycin as preservatives on it and keeping it between two glass plates. It was then kept in the refrigerator and used within two three days.

The skin permeation of estradiol, ethinylestradiol, and levonorgestrel from proniosome formulations was determined by using Franze diffusion cell (locally fabricated). The rats' skin was mounted on the receptor compartment with stratum corneum side facing upwards into the donor compartment. The top of the diffusion cell was covered with paraffin paper. The donor compartment was filled with proniosomal gel (100mg). A 10 ml aliquot of 40% PEG v/v 200 was used as receptor medium to maintain the sink condition. The available permeated area was 1.13cm^2. The receptor compartment was maintained at 37°C and stirred by a magnetic bar at 400-500 rpm. At appropriate intervals 5.0 ml of aliquots of the receptor fluid were withdrawn and immediately replaced by an equal volume of fresh receptor fluid. The samples withdrawn were then analyzed spectrophotometrically using UV Shimadzu-1700 spectrophotometer, against respective blank and amount of drug diffused at various time intervals was determined from standard curve calculation data through multi-component mode, spectrophotometry for combination. In vitro release rate studies were performed for different formulations and effect of various composition, spans, alcohols and sonication on release rate were studies. The observations are recorded in Table 4.14-4.15.

Fabrication of Transdermal Patch:

The circular ring of flexible fiber having thickness 0.9 mm, outer diameter 1.35 cm and inner diameter 1.25 cm was stuck on the adhesive tape. A circular aluminium foil of diameter 1.50 cm, was used as backing membrane and kept on the ring with the little pressure to prepare reservoir cavity. The inner diameter of cavity was 1.20 cm (corresponding to 1.13 cm^2.area). The proniosomal gel was evenly spread into this reservoir cavity and covered with a thin nylon mesh (Jhonson and Jhonson). Finally round shaped patch was stuck on a polythene foil, which was removed before use and applied on the depilated skin area on the neck of rats. The fabricated transdermal patches were then used for the *in vivo* studies in female rats.

Table 4.3: Permeation profile of Ethinylestradiol form proniosomal gel formulation containing Span & tween (Surfactant ratio) through the cellophane membrane (Span $_{20}$:Tween $_{20:40:60:80}$)

S. No.	Time (Hrs)	Cumulative drug permeated from formulations (µg/cm^2)			
		S20(3):T20(1)	S20 (3): T40(1)	S20(3):T60(1)	S20(3):T80(1)
1.	0.75	31.66 ± 1.14	24.49 ± 2.63	23.85 ± 4.71	27.81 ± 4.11
2.	1.50	61.16 ± 1.98	49.71 ± 3.21	46.84 ± 5.25	53.20 ± 3.56
3.	2.25	92.51 ± 2.24	75.58 ± 1.94	68.95 ± 6.98	80.75 ± 5.66
4.	3.00	123.51 ± 3.24	101.57 ± 2.23	92.75 ± 5.54	108.19 ± 4.33
5.	3.75	154.83 ± 4.87	126.56 ± 3.47	117.08 ± 6.82	136.63 ± 7.88
6.	4.50	184.71 ± 5.47	150.55 ± 5.54	141.96 ± 9.11	164.76 ± 8.99
7.	5.25	216.37 ± 6.67	174.32 ± 6.32	165.92 ± 10.22	192.74 ± 10.01
8.	6.00	248.81 ± 5.89	200.33 ± 7.01	187.91 ± 11.02	221.88 ± 8.77
9.	6.75	281.84 ± 6.66	226.75 ± 6.98	208.99 ± 6.33	250.21 ± 9.88
10.	7.50	316.35 ± 7.41	249.89 ± 8.99	231.78 ± 7.77	278.87 ± 3.55
11.	8.25	350.12 ± 8.81	283.87 ± 3.99	264.46 ± 8.44	306.86 ± 6.44
12.	24.00	393.98 ± 9.91	330.40 ± 4.77	299.66 ± 4.66	349.63 ±10.77

Table 4.4: Permeation profile of Ethinylestradiol form proniosomal gel formulation containing Span & tween (Surfactant ratio) through the cellophane membrane (Span $_{40(1,2,3,4)}$: Tween $_{20}$)

S. No.	Time (Hrs)	Cumulative drug permeated from formulations (µg/cm^2)			
		S40(1): T20(1)	S40(2):T20(1)	S40(3): T20(1)	S40(4): T20(1)
1.	0.75	22.79 ± 0.99	21.12 ± 2.88	17.44 ± 1.7	15.42 ± 1.33
2.	1.50	46.59 ± 1.54	44.14 ± 4.44	35.66 ± 2.66	31.11 ± 2.67
3.	2.25	71.50 ± 3.33	65.43 ± 3.33	53.65 ± 6.55	48.44 ± 3.45
4.	3.00	98.72 ± 4.21	86.98 ± 1.69	72.31 ± 5.44	66.05 ± 6.55
5.	3.75	129.83 ± 4.88	108.64 ± 4.81	90.86 ± 6.17	87.16 ± 5.61
6.	4.50	156.94 ± 5.66	129.63 ± 5.64	109.52 ± 6.88	107.15 ± 5.11
7.	5.25	181.82 ± 7.31	151.62 ± 3.99	128.74 ± 7.44	123.14 ± 2.99
8.	6.00	206.70 ± 3.89	174.50 ± 1.77	150.07 ± 4.31	139.80 ± 4.71
9.	6.75	230.69 ± 8.41	196.53 ± 8.88	171.84 ± 3.61	156.92 ± 7.33
10.	7.50	257.02 ± 8.99	218.61 ± 7.96	194.92 ± 9.88	174.04 ± 8.66
11.	8.25	284.68 ± 10.11	242.60 ± 6.98	228.83 ± 7.11	201.26 ± 7.01
12.	24.00	323.92 ± 9.77	285.84 ± 7.44	274.82 ± 6.88	240.52 ± 10.22

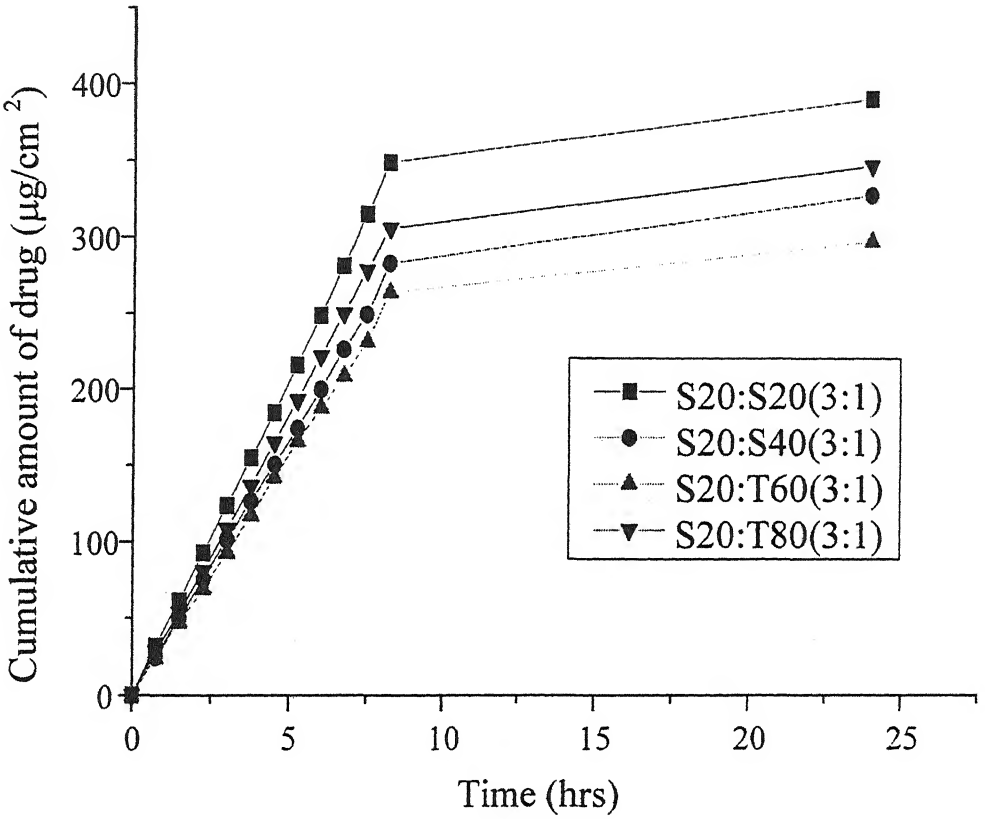


Figure 4.7: Permeation profile of Ethinylestradiol form proniosomal gel formulation containing Span & tween (Surfactant ratio) through the cellophane membrane (Span $_{20}$:Tween $_{20:40:60:80}$)

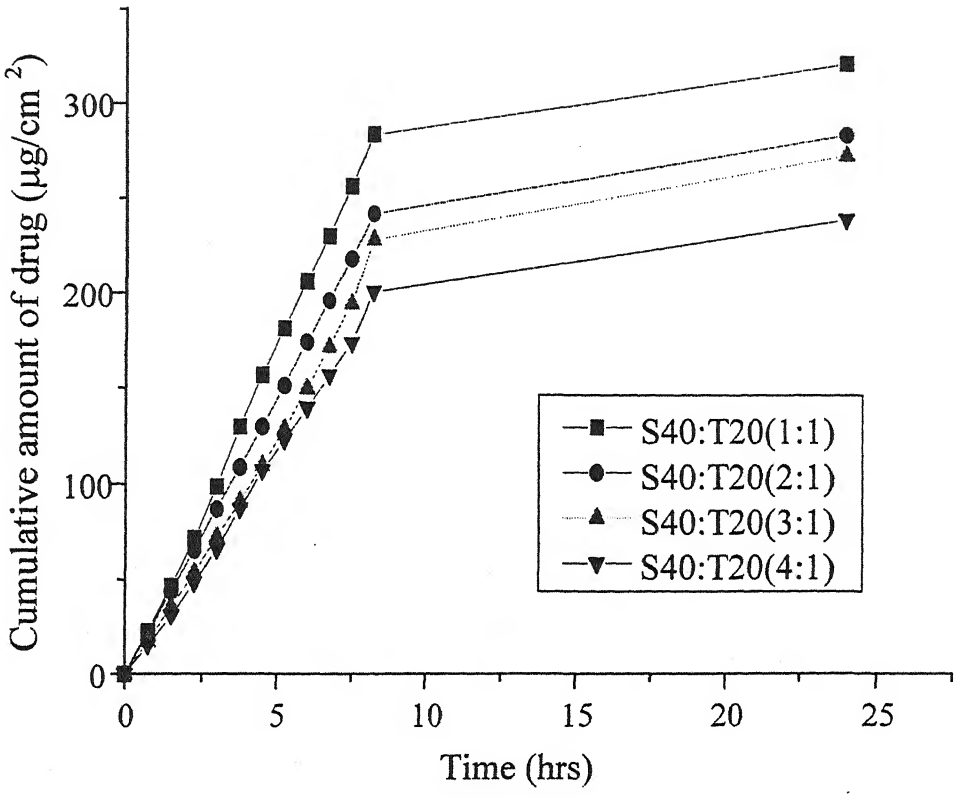


Figure 4.8: Permeation profile of Ethinylestradiol form proniosomal gel formulation containing Span & tween (Surfactant ratio) through the cellophane membrane (Span $_{40(1,2,3,4)}$: Tween $_{20}$)

Table 4.5: Permeation profile of Ethinylestradiol form proniosomal gel formulation containing Span & tween (Surfactant ratio) through the cellophane membrane (Span $_{40}$:Tween $_{20:40:60:80}$)

S. No.	Time (Hrs)	Cumulative drug permeated from formulations (μg/cm^2)			
		S40(3):T20(1)	S40(3):T40(1)	S40(3): T60(1)	S40(3): T80(1)
1.	0.75	22.49 ± 1.11	18.21 ± 2.61	20.71 ± 1.17	19.41 ± 0.98
2.	1.50	52.04 ± 2.33	36.43 ± 1.82	37.83 ± 2.47	38.41 ± 1.63
3.	2.25	83.70 ± 2.46	54.31 ± 3.75	55.05 ± 3.75	57.39 ± 4.12
4.	3.00	103.58 ± 3.74	71.52 ± 6.38	70.54 ± 3.99	77.27 ± 3.74
5.	3.75	122.54 ± 3.88	88.63 ± 5.94	86.65 ± 4.19	97.28 ± 6.48
6.	4.50	141.14 ± 6.10	105.62 ± 6.11	103.53 ± 6.81	118.67 ± 6.94
7.	5.25	159.66 ± 5.18	125.42 ± 7.32	119.75 ± 4.34	139.33 ± 7.70
8.	6.00	178.65 ± 6.73	146.68 ± 4.50	135.31 ± 6.87	158.74 ± 8.06
9.	6.75	195.64 ± 5.87	165.91 ± 4.11	161.18 ± 6.91	179.99 ± 10.74
10.	7.50	210.74 ± 6.74	188.89 ± 8.19	177.40 ± 3.80	202.19 ± 11.13
11.	8.25	231.52 ± 6.91	209.88 ± 6.14	193.04 ± 7.63	223.73 ± 8.91
12.	24.00	278.84 ± 8.13	251.43 ± 11.31	240.54 ± 9.32	269.88 ± 9.09

Table 4.6: Permeation profile of Ethinylestradiol form proniosomal gel formulation containing Span & tween (Surfactant ratio) through the cellophane membrane (Span $_{60}$:Tween $_{20:40:60:80}$)

S. No.	Time (Hrs)	Cumulative drug permeated from formulations (μg/cm^2)			
		S60(3):T20(1)	S60(3):T40(1)	S60(3): T60(1)	S60(3): T80(1)
1.	0.75	13.47 ± 1.12	10.94 ± 1.77	10.05 ± 2.11	11.44 ± 3.30
2.	1.50	32.91 ± 2.21	27.53 ± 2.46	25.41 ± 6.33	29.77 ± 11.11
3.	2.25	52.13 ± 3.14	44.64 ± 3.74	39.46 ± 4.55	47.99 ± 3.46
4.	3.00	76.13 ± 3.11	61.63 ± 4.12	54.69 ± 7.66	66.54 ± 3.55
5.	3.75	95.12 ± 4.11	78.29 ± 2.21	71.25 ± 2.33	85.20 ± 6.53
6.	4.50	114.21 ± 5.41	94.28 ± 6.44	88.25 ± 7.41	103.42 ± 7.49
7.	5.25	132.76 ± 5.55	111.72 ± 7.21	103.94 ± 6.57	121.64 ± 5.68
8.	6.00	152.75 ± 6.11	129.66 ± 8.11	119.16 ± 7.39	139.97 ± 8.46
9.	6.75	172.08 ± 6.01	146.82 ± 6.56	135.49 ± 4.89	158.63 ± 6.43
10.	7.50	192.32 ± 7.12	163.93 ± 7.48	152.33 ± 8.12	187.07 ± 7.33
11.	8.25	212.54 ± 5.44	181.59 ± 9.47	167.58 ± 6.47	205.51 ± 9.16
12.	24.00	258.42 ± 9.98	220.58 ± 10.33	206.02 ± 8.41	243.95 ± 8.42

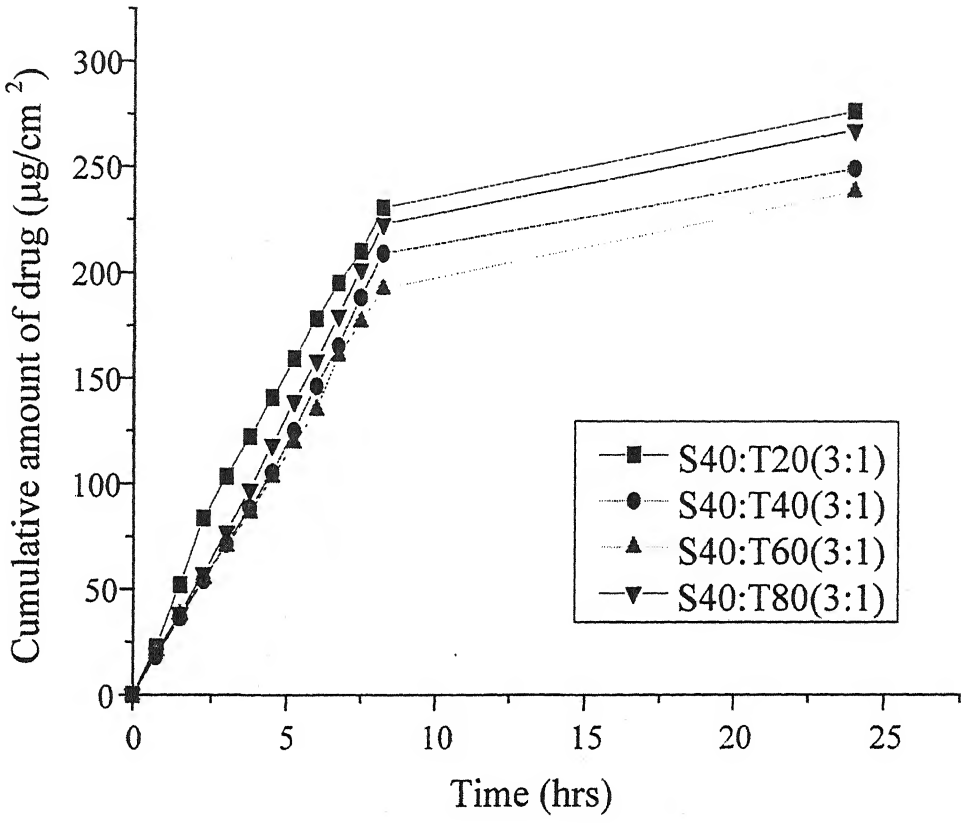


Figure 4.9: Permeation profile of Ethinylestradiol form proniosomal gel formulation containing Span & tween (Surfactant ratio) through the cellophane membrane (Span $_{40}$:Tween $_{20:40:60:80}$)

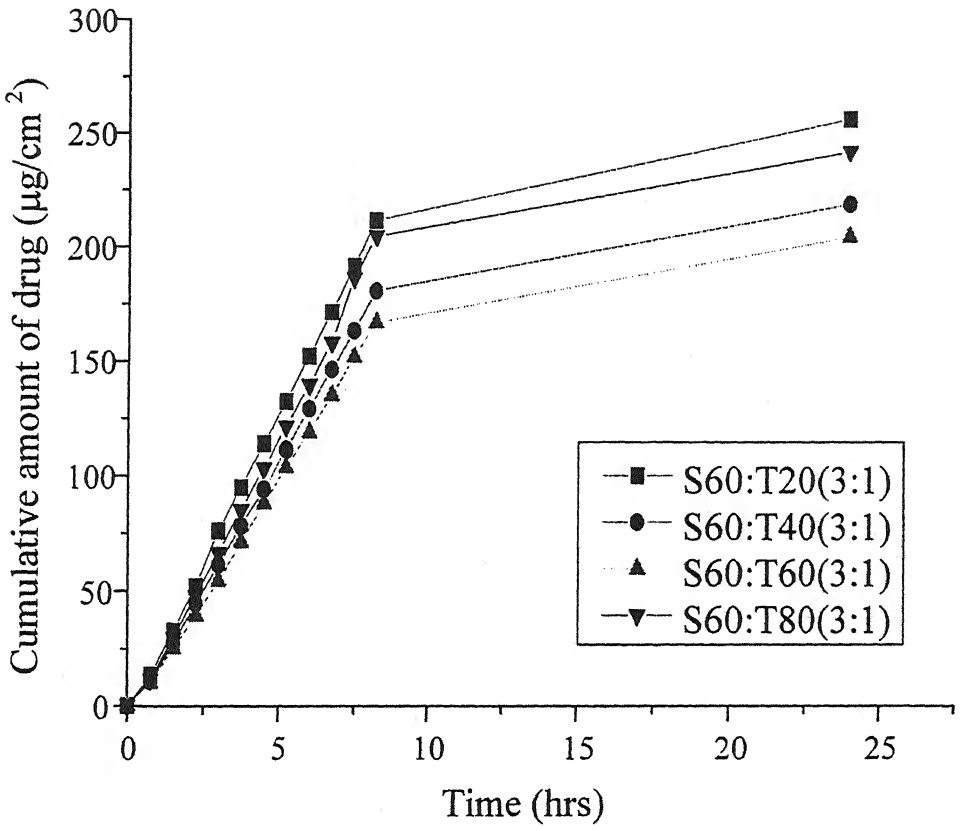


Figure 4.10: Permeation profile of Ethinylestradiol form proniosomal gel formulation containing Span & tween (Surfactant ratio) through the cellophane membrane (Span $_{60}$:Tween $_{20:40:60:80}$)

Table 4.7: Permeation profile of Ethinylestradiol form proniosomal gel formulation containing Span & tween (Surfactant ratio) through the cellophane membrane (Span $_{80}$:Tween $_{20:40:60:80}$)

S. No.	Time (Hrs)	Cumulative drug permeated from formulations (μg/cm^2)			
		S80(3):T20(1)	S80(3):T40(1)	S80(3):T60(1)	S80(3):T80(1)
1.	0.75	26.20 ± 1.37	22.60 ± 3.31	22.05 ± 1.69	23.64 ± 0.98
2.	1.50	50.46 ± 2.31	45.28 ± 4.36	44.26 ± 2.33	47.07 ± 1.11
3.	2.25	69.42 ± 4.63	68.43 ± 4.12	66.57 ± 4.71	71.21 ± 2.62
4.	3.00	99.66 ± 5.77	92.19 ± 6.37	90.03 ± 8.38	93.02 ± 3.41
5.	3.75	130.53 ± 8.12	116.00 ± 5.01	113.87 ± 2.45	115.92 ± 4.82
6.	4.50	155.54 ± 7.36	142.89 ± 5.74	137.34 ± 3.98	139.40 ± 3.11
7.	5.25	177.82 ± 4.36	169.91 ± 6.07	159.94 ± 4.89	162.00 ± 7.61
8.	6.00	213.42 ± 7.83	198.12 ± 6.13	181.99 ± 5.73	186.80 ± 3.54
9.	6.75	251.79 ± 9.61	226.48 ± 2.88	205.70 ± 6.17	230.64 ± 8.54
10.	7.50	282.32 ± 6.74	255.03 ± 6.99	229.35 ± 6.97	265.88 ± 9.59
11.	8.25	313.42 ± 6.07	283.81 ± 5.88	254.48 ± 8.20	302.42 ± 7.41
12.	24.00	364.12 ± 8.39	332.73 ± 7.18	300.42 ± 6.77	342.64 ± 10.74

Table 4.8: Permeation profile of Ethinylestradiol form proniosomal gel formulation containing Spans, Span & tween (Surfactant ratio) through the cellophane membrane (Span $_{40:20}$ & Span $_{40}$:Tween $_{20:40:60:80}$)

S. No.	Time (Hrs)	Cumulative drug permeated from formulations (μg/cm^2)				
		S40(3):S20(1)	S40(1):T20(1)	S40(2):T20(1)	S40(3):T20(1)	S40(4):T20(1)
1.	0.75	17.46 ± 3.13	22.79 ± 1.54	21.12 ± 4.11	12.44 ± 1.32	15.42 ± 2.67
2.	1.50	33.89 ± 1.22	46.59 ± 3.87	44.14 ± 6.33	35.66 ± 4.63	31.11 ± 2.99
3.	2.25	51.01 ± 3.83	71.50 ± 5.25	65.43 ± 4.44	53.65 ± 3.58	48.44 ± 6.47
4.	3.00	67.78 ± 4.17	98.72 ± 3.35	86.98 ± 2.87	72.31 ± 4.72	66.05 ± 5.42
5.	3.75	83.66 ± 6.46	129.83 ± 4.64	108.64 ± 3.92	90.86 ± 6.82	87.16 ± 2.47
6.	4.50	100.01 ± 2.88	156.94 ± 2.78	129.63 ± 4.84	109.52 ± 6.47	107.15 ± 6.81
7.	5.25	108.14 ± 9.74	181.82 ± 6.45	151.62 ± 2.66	128.74 ± 2.74	123.14 ± 7.26
8.	6.00	136.58 ± 7.21	206.70 ± 8.12	174.50 ± 7.87	150.07 ± 6.89	139.80 ± 7.03
9.	6.75	157.02 ± 3.45	230.69 ± 10.44	196.53 ± 8.23	171.84 ± 5.87	146.92 ± 4.68
10.	7.50	176.48 ± 9.72	257.02 ± 9.84	218.61 ± 4.65	194.92 ± 6.47	174.04 ± 8.31
11.	8.25	193.81 ± 6.71	284.68 ± 8.38	242.60 ± 7.95	228.83 ± 3.98	201.26±10.87
12.	24.00	224.05 ± 8.75	333.92 ± 5.87	295.84 ± 8.70	292.82±10.45	250.52 ± 9.81

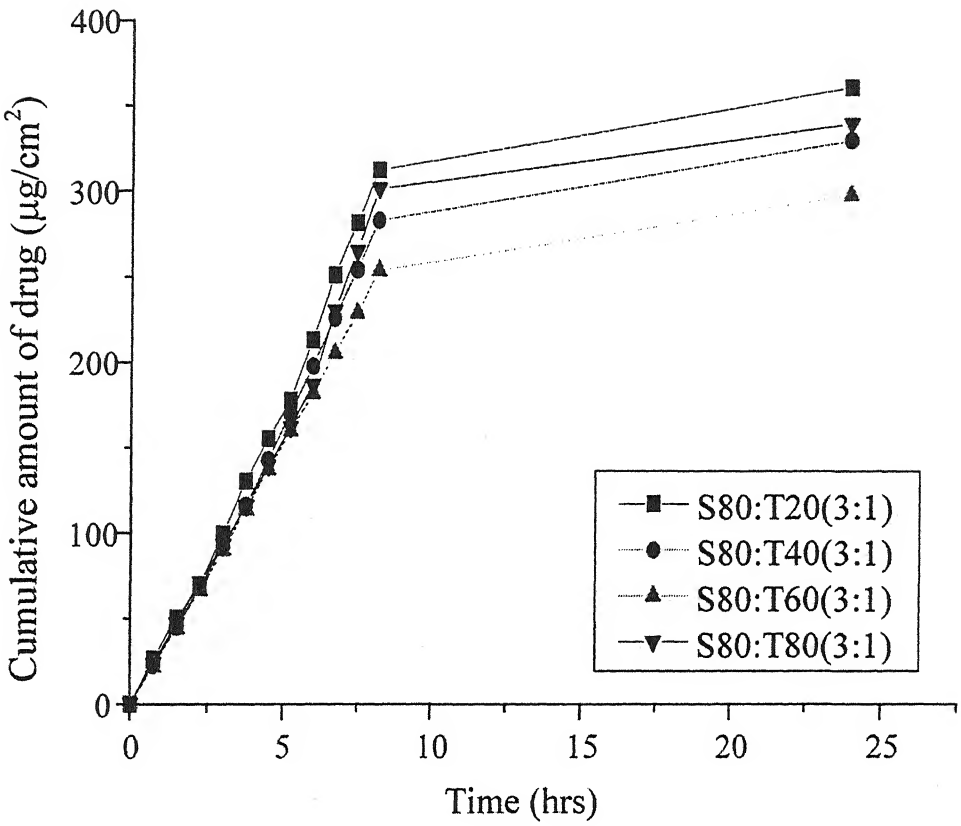


Figure 4.11:Permeation profile of Ethinylestradiol form proniosomal gel formulation containing Span & tween (Surfactant ratio) through the cellophane membrane (Span $_{80}$:Tween $_{20:40:60:80}$)

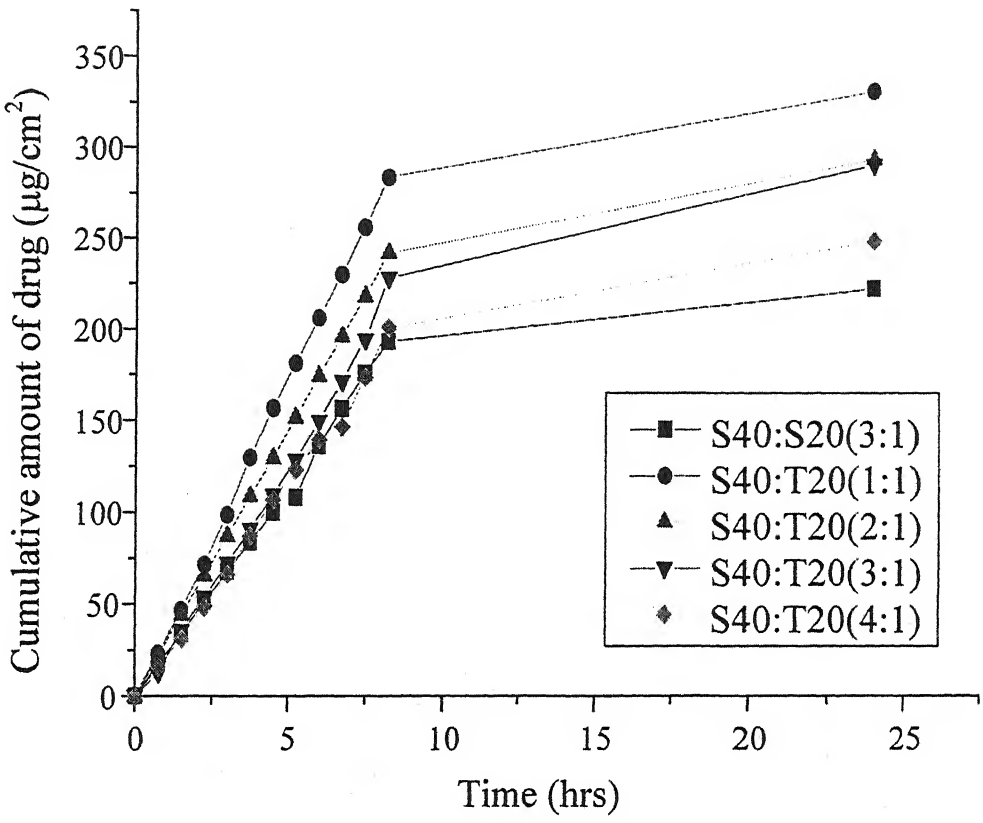


Figure 4.12: Permeation profile of Ethinylestradiol form proniosomal gel formulation containing Spans, Span & tween (Surfactant ratio) through the cellophane membrane (Span $_{40:20}$ & Span $_{40}$:Tween $_{20:40:60:80}$)

Table 4.9: Effect of different alcohols on permeation profile of Ethinylestradiol form proniosomal gel formulation containing Span & tween (Surfactant ratio) through the cellophane membrane

S. No.	Time (Hrs)	Cumulative drug permeated from formulations (μg/cm²)				
		S40(3):S20(1)	Isopropyl alcohol S40(3):T20(1)	Absolute alcohol S40(3):T20(1)	Propanol S40(3):T20(1)	Butanol S40(3):T20(1)
1.	0.75	17.46 ± 3.13	24.49 ± 1.12	23.12 ± 1.25	21.00 ± 3.78	14.99 ± 0.94
2.	1.50	33.89 ± 1.22	49.71 ± 3.31	44.41 ± 3.74	41.12 ± 5.41	35.12 ± 1.84
3.	2.25	51.01 ± 3.83	75.58 ± 2.47	66.66 ± 3.89	62.18 ± 6.34	56.77 ± 2.82
4.	3.00	67.78 ± 4.17	101.57 ± 5.61	89.75 ± 6.65	83.15 ± 3.30	76.65 ± 4.39
5.	3.75	83.66 ± 6.46	126.56 ± 6.23	114.82 ± 6.65	104.12 ± 3.99	97.64 ± 5.47
6.	4.50	100.01 ± 2.88	150.55 ± 8.47	136.05 ± 4.87	125.33 ± 4.88	116.49 ± 2.64
7.	5.25	108.14 ± 9.74	174.32 ± 4.69	159.34 ± 7.85	146.44 ± 5.64	136.50 ± 7.46
8.	6.00	136.58 ± 7.21	200.33 ± 2.83	179.35 ± 9.65	168.44 ± 3.98	155.48 ± 8.11
9.	6.75	157.02 ± 3.45	226.75 ± 7.89	200.74 ± 4.63	189.43 ± 7.75	173.47 ± 7.36
10.	7.50	176.48 ± 9.72	249.89 ± 10.40	225.66 ± 3.77	210.09 ± 8.61	193.91 ± 8.49
11.	8.25	193.81 ± 6.71	283.87 ± 4.46	256.78 ± 9.70	231.08 ± 9.74	214.79 ± 6.18
12.	24.00	224.05 ± 8.75	340.40 ± 9.83	319.34 ± 10.60	275.44 ± 7.46	256.56 ± 6.99

Table 4.10: Effect of sonication time on Permeation profile of Ethinylestradiol form proniosomal gel formulation containing Span & tween (Surfactant ratio) through the cellophane membrane

S. No.	Time (Hrs)	Cumulative drug permeated from formulations (μg/cm²)				
		S40(3):T20(1)	30Sec	60Sec	90Sec	120Sec
1.	0.75	24.49 ± 1.12	26.33 ± 1.73	29.36 ± 1.45	34.33 ± 1.99	39.42 ± 1.22
2.	1.50	49.71 ± 3.31	52.32 ± 2.45	58.16 ± 3.41	69.32 ± 6.44	78.64 ± 8.20
3.	2.25	75.58 ± 2.47	79.31 ± 6.33	85.72 ± 6.23	104.31 ± 3.45	117.52 ± 10.40
4.	3.00	101.57 ± 5.61	105.19 ± 4.15	113.56 ± 5.84	139.63 ± 4.78	155.18 ± 4.55
5.	3.75	126.56 ± 6.23	130.18 ± 6.54	137.44 ± 5.99	175.85 ± 9.63	183.29 ± 1.99
6.	4.50	150.55 ± 8.47	157.51 ± 4.82	166.43 ± 6.44	213.84 ± 2.88	229.84 ± 7.46
7.	5.25	174.32 ± 4.69	185.74 ± 9.88	195.63 ± 7.17	247.83 ± 1.79	267.50 ± 6.41
8.	6.00	200.33 ± 2.83	212.22 ± 4.73	221.14 ± 7.63	282.27 ± 6.87	307.38 ± 3.37
9.	6.75	226.75 ± 7.89	238.88 ± 5.61	245.29 ± 8.15	316.15 ± 8.42	347.93 ± 4.89
10.	7.50	249.89 ± 10.40	265.65 ± 8.44	276.14 ± 6.41	351.69 ± 7.38	390.59 ± 8.49
11.	8.25	283.87 ± 4.46	295.13 ± 10.43	306.39 ± 6.87	386.33 ± 4.86	428.25 ± 8.17
12.	24.00	340.40 ± 9.83	350.87 ± 8.63	362.38 ± 8.09	440.32 ± 6.48	487.24 ± 9.59

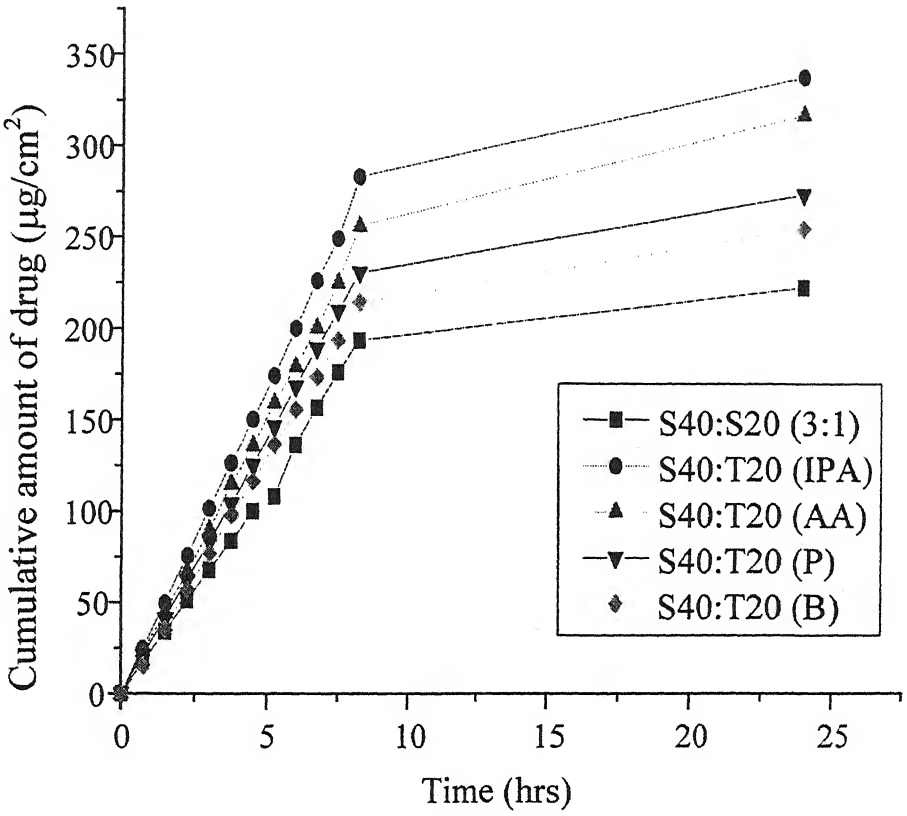


Figure 4.13: Effect of different alcohols on permeation profile of Ethinylestradiol form proniosomal gel formulation containing Span & tween (Surfactant ratio) through the cellophane membrane

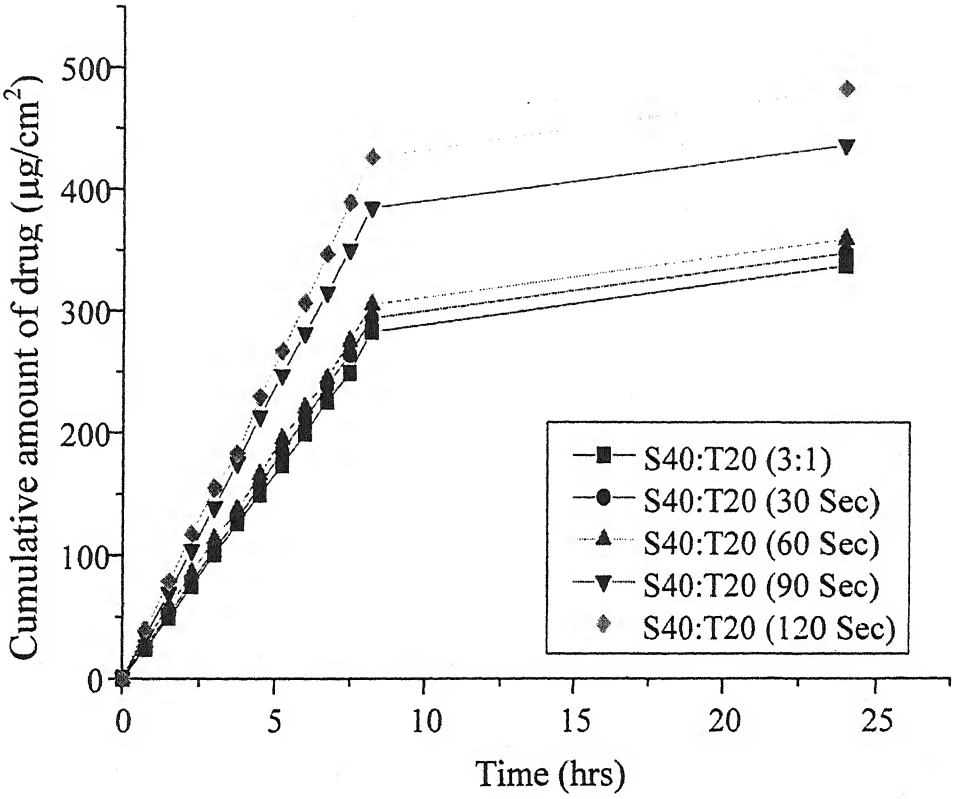


Figure 4.14: Effect of sonication on Permeation profile of Ethinylestradiol form proniosomal gel formulation containing Span & tween (Surfactant ratio) through the cellophane membrane

Table 4.11: Effect of lipids on drug permeation of Ethinylestradiol form proniosomal gel formulation containing Span & tween with lipids through the cellophane membrane (Span $_{40}$:Tween $_{20:\ EL:\ SL:\ DCP:\ CHL}$)

S. No.	Time (Hrs)	Cumulative drug permeated from formulations (μg/cm^2)				
		S40(3):T20(1)	S40:T20:EL (3:1:1)	S40:T20:SL (3:1:1)	S40:T20:DCP (3:1:1)	S40:T40:CHL (3:1:1)
1.	0.75	24.49 ± 1.12	27.75 ± 2.41	31.78 ± 3.46	30.75 ± 1.01	23.94 ± 1.63
2.	1.50	49.71 ± 3.31	55.02 ± 3.79	69.33 ± 2.81	60.41 ± 1.99	48.27 ± 3.46
3.	2.25	75.58 ± 2.47	83.31 ± 6.81	99.79 ± 6.31	91.64 ± 6.33	73.38 ± 4.51
4.	3.00	101.57 ± 5.61	110.03 ± 5.46	132.68 ± 2.30	211.89 ± 4.12	98.04 ± 8.12
5.	3.75	126.56 ± 6.23	138.90 ± 5.99	165.56 ± 3.54	151.45 ± 5.31	125.75 ± 4.31
6.	4.50	150.55 ± 8.47	166.78 ± 6.77	197.55 ± 3.64	181.11 ± 3.82	151.37 ± 3.68
7.	5.25	174.32 ± 4.69	194.54 ± 6.99	231.54 ± 4.17	211.80 ± 7.61	182.07 ± 4.17
8.	6.00	200.33 ± 2.83	221.67 ± 5.81	264.52 ± 4.92	242.24 ± 6.98	205.25 ± 5.49
9.	6.75	226.75 ± 7.89	248.31 ± 8.13	298.74 ± 4.62	271.76 ± 4.83	234.58 ± 2.66
10.	7.50	249.89±10.40	274.64 ± 3.99	329.73 ± 7.28	301.62 ± 8.09	268.91 ± 3.44
11.	8.25	283.87±4.46	304.06 ± 10.10	364.61 ± 6.61	333.08±10.91	293.79 ± 10.81
12.	24.00	340.40 ± 9.83	354.05 ± 5.83	421.16 ± 8.91	382.15±11.09	348.12 ± 7.36

Table 4.12: Permeation profile of Estradiol form proniosomal gel formulation containing Span & tween (Surfactant ratio) through the cellophane membrane (Span$_{40}$: Tween$_{20:40:60:80}$)

S. No.	Time (Hrs)	Cumulative drug permeated from formulations (μg/cm^2)			
		S40(1):T20(1)	S40(2):T40(1)	S40(3):T60(1)	S40(3):T80(1)
1.	0.75	23.44 ± 4.62	18.65 ± 1.96	16.60 ± 0.95	20.66 ± 6.11
2.	1.50	49.86 ± 2.14	39.20 ± 1.82	36.15 ± 1.78	45.65 ± 3.12
3.	2.25	75.97 ± 7.12	60.19 ± 4.36	54.81 ± 6.32	69.42 ± 7.43
4.	3.00	102.03 ± 6.84	81.74 ± 5.48	72.08 ± 4.32	93.88 ± 8.12
5.	3.75	139.86 ± 6.99	103.51 ± 6.80	91.45 ± 3.34	118.63 ± 6.32
6.	4.50	156.41 ± 4.88	125.84 ± 7.22	110.11 ± 7.78	143.29 ± 4.36
7.	5.25	194.63 ± 6.12	159.28 ± 4.66	129.34 ± 3.84	167.28 ± 7.86
8.	6.00	220.96 ± 7.12	173.83 ± 2.99	150.33 ± 8.43	191.96 ± 10.11
9.	6.75	258.07 ± 2.15	197.38 ± 7.64	171.77 ± 5.66	217.29 ± 10.40
10.	7.50	296.73 ± 3.93	222.07 ± 8.19	193.33 ± 4.94	243.52 ± 8.31
11.	8.25	316.29 ± 7.16	249.06 ± 4.91	213.76 ± 7.16	269.96 ± 4.62
12.	24.00	368.18 ± 6.06	298.51 ± 5.94	263.75 ± 6.00	319.84 ± 10.77

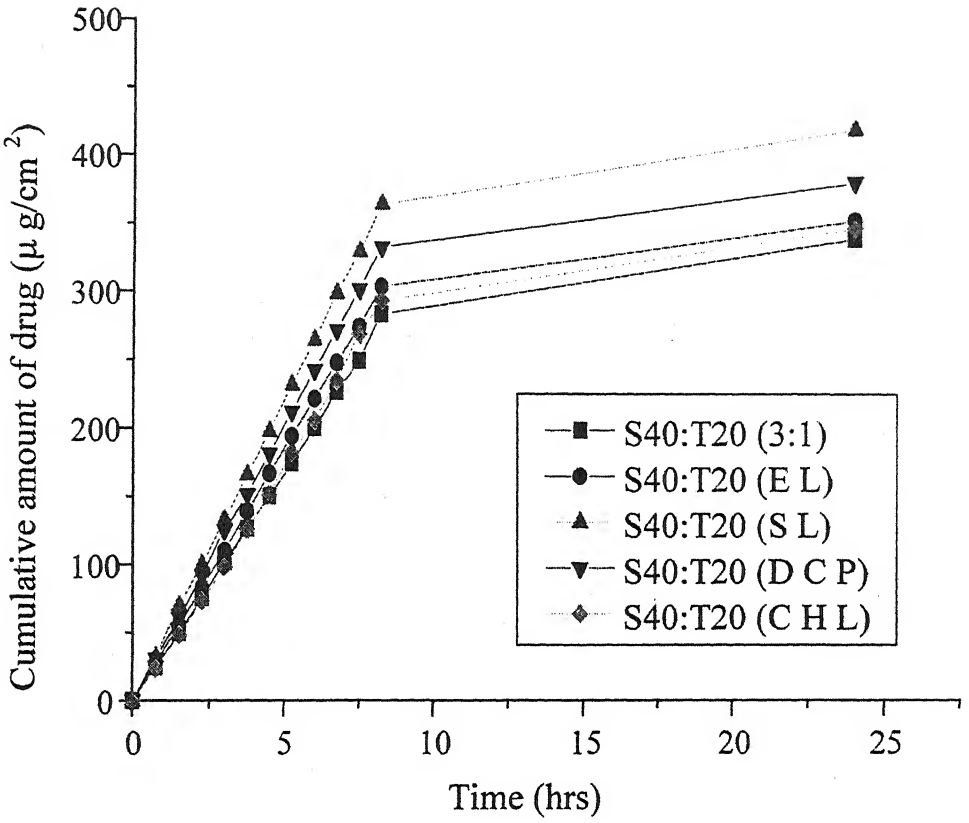


Figure 4.15: Effect of lipids on drug permeation of Ethinylestradiol form proniosomal gel formulation containing Span & tween with lipids through the cellophane membrane (Span $_{40}$:Tween $_{20}$: EL : SL : DCP :CHL)

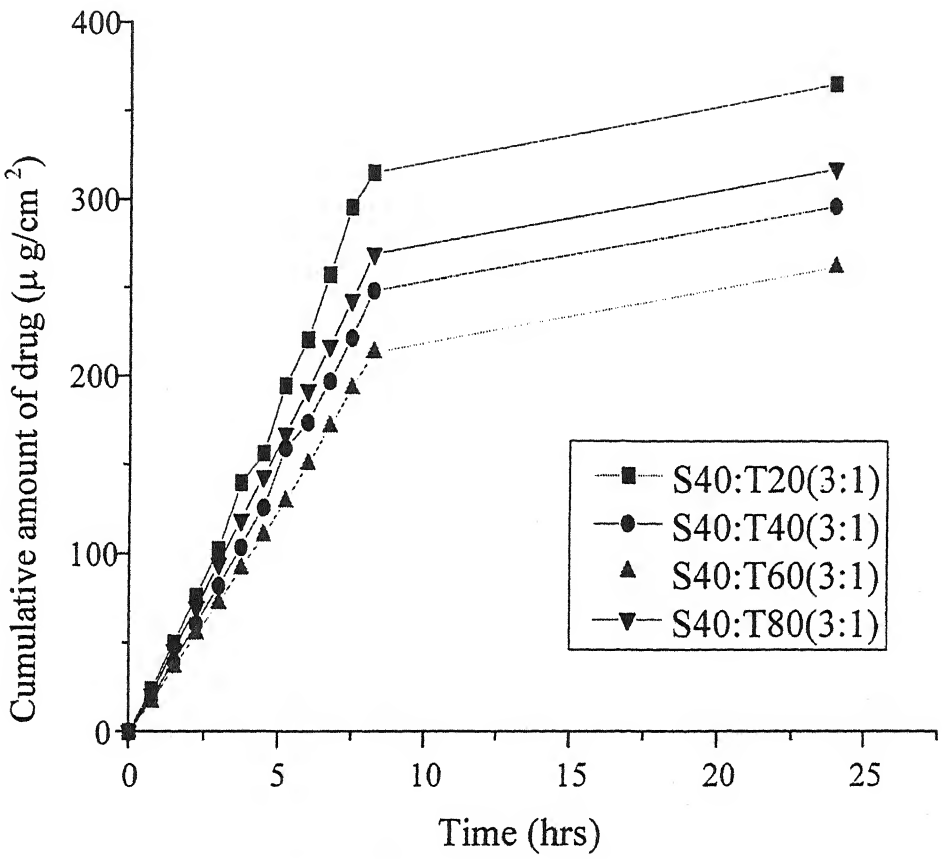


Figure 4.16: Permeation profile of Estradiol form proniosomal gel formulation containing Span & tween (Surfactant ratio) through the cellophane membrane (Span $_{40}$: Tween $_{20:40:60:80}$)

Table 4.13: Permeation profile of Levonorgestrel form proniosomal gel formulation containing Span & tween (Surfactant ratio) through the cellophane membrane (Span $_{40}$:Tween $_{20:40:60:80}$)

S. No.	Time (Hrs)	Cumulative drug permeated from formulations (μg/cm^2)			
		S40(1):T20(1)	S40(2):T40(1)	S40(3):T60(1)	S40(3):T80(1)
1.	0.75	16.11 ± 3.30	13.84 ± 0.89	14.54 ± 1.63	14.99 ± 1.44
2.	1.50	34.82 ± 2.66	30.96 ± 1.63	29.33 ± 2.34	34.62 ± 2.81
3.	2.25	50.64 ± 4.11	48.29 ± 4.23	44.21 ± 1.97	55.61 ± 3.64
4.	3.00	72.95 ± 7.82	67.17 ± 2.93	59.42 ± 3.99	71.16 ± 4.52
5.	3.75	91.66 ± 6.13	86.61 ± 4.84	75.65 ± 4.68	89.60 ± 5.28
6.	4.50	119.91 ± 2.84	105.17 ± 6.69	93.76 ± 4.11	110.59 ± 2.99
7.	5.25	152.81 ± 7.99	123.83 ± 7.12	112.92 ± 10.73	131.18 ± 3.89
8.	6.00	190.21 ± 8.32	143.06 ± 8.46	131.56 ± 1.85	140.84 ± 7.94
9.	6.75	229.41 ± 4.62	161.05 ± 9.12	151.11 ± 11.20	171.83 ± 4.61
10.	7.50	269.36 ± 4.68	180.60±10.19	171.10 ± 8.99	193.49 ± 10.33
11.	8.25	297.51 ± 7.12	203.26 ± 7.82	195.07 ± 8.66	215.37 ± 11.41
12.	24.00	348.21 ± 8.93	248.14 ± 7.99	242.18 ± 9.08	258.15 ± 7.19

Table 4.14: Permeation profile of Ethinylestradiol+Levonorgestrel from optimized proniosomal gel containing Spans, Span & Tween (Surfactant ratio) through the rat skin; Span $_{40}$: Span $_{20}$ (S1) and Span$_{40}$: Tween $_{20}$ (T1)

S.No.	Time (Hrs)	Cumulative drug permeated from formulations (μg/cm^2)			
		S40(3): S20(1) (Code-S1)		S40(3):T20(1) (Code-T1)	
1.	0.75	6.19 ± 0.69	3.43 ± 1.28	6.43 ± 1.37	4.42 ± 1.68
2.	1.50	12.62 ± 1.32	9.52 ± 3.33	13.48 ± 1.55	10.41 ± 3.81
3.	2.25	19.64 ± 2.88	15.70 ± 1.28	23.46 ± 6.32	17.06 ± 5.45
4.	3.00	26.69 ± 3.66	21.77 ± 4.63	30.63 ± 4.18	24.79 ± 6.23
5.	3.75	35.46 ± 7.45	30.53 ± 5.46	44.85 ± 6.34	33.78 ± 4.74
6.	4.50	48.92 ± 6.12	39.30 ± 3.48	58.51 ± 7.14	43.77 ± 5.23
7.	5.25	59.28 ± 3.46	50.19 ± 4.56	72.06 ± 6.48	55.88 ± 3.46
8.	6.00	74.11 ± 7.12	61.54 ± 4.63	86.72 ± 2.93	71.87 ± 4.74
9.	6.75	99.12 ± 6.41	78.38 ± 7.85	102.38 ± 4.99	89.86 ± 4.23
10.	7.50	113.35 ± 1.99	89.68 ± 7.09	118.04 ± 10.17	107.03 ± 7.71
11.	8.25	126.54 ± 8.74	104.67 ± 8.12	134.91 ± 8.16	126.51 ± 8.62
12.	24.00	151.42 ± 10.79	129.44 ± 6.98	165.99 ± 7.73	158.72 ± 10.42

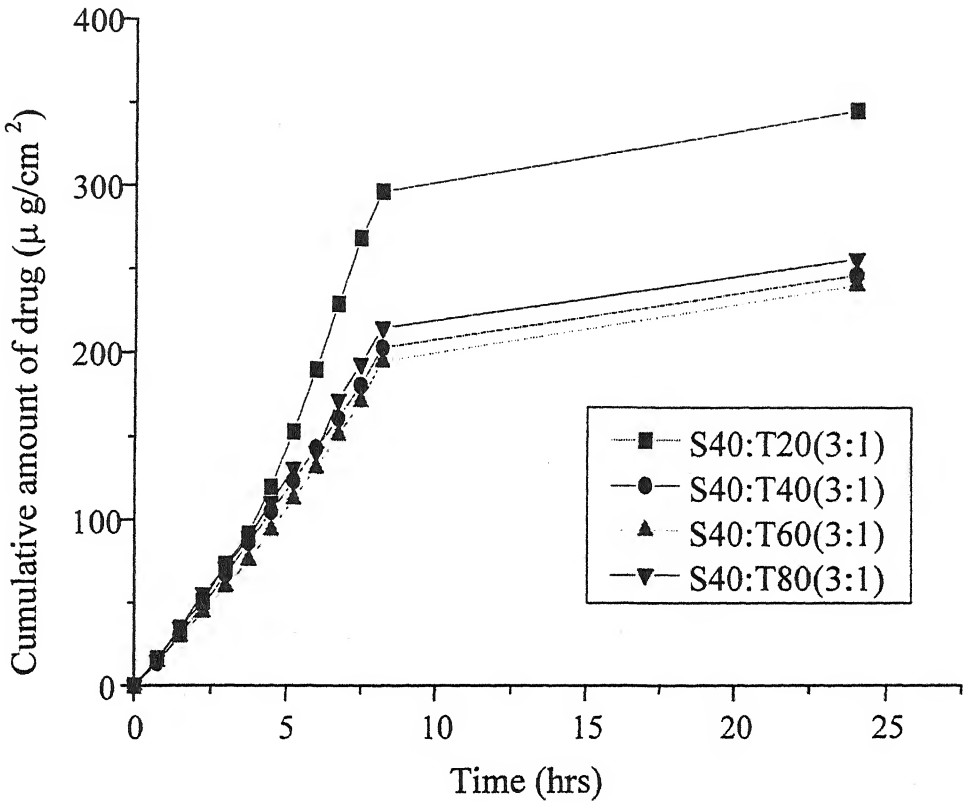


Figure 4.17: Permeation profile of Levonorgestrel form proniosomal gel formulation containing Span & tween (Surfactant ratio) through the cellophane membrane (Span $_{40}$: Tween $_{20:40:60:80}$)

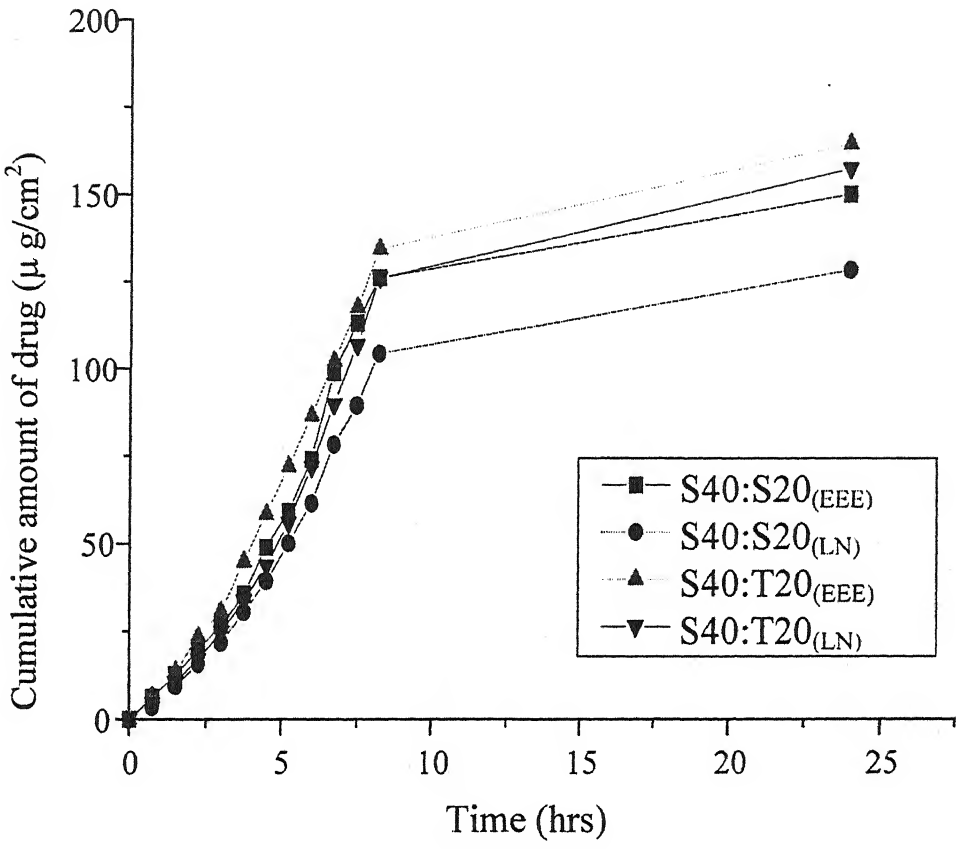


Figure 4.18: Permeation profile of Ethinylestradiol+Levonorgestrel from optimized proniosomal gel containing Spans, Span & Tween (Surfactant ratio) through the rat skin; Span $_{40}$: Span $_{20}$ (S1) and Span$_{40}$: Tween $_{20}$ (T1)

Table 4.15: Permeation profile of Estradiol+Levonorgestrel from optimized proniosomal gel containing Spans, Span & Tween (Surfactant ratio) through rat skin; Span $_{40}$: Span $_{20}$ (S2) and Span$_{40}$: Tween $_{20}$ (T2)

S.No.	Time (Hrs)	Cumulative drug permeated from formulations (μg/cm^2)			
		S40 : S20 (3:1) (Code-S2)		S40 : T20 (3:1) (Code-T2)	
1.	0.75	6.95 ± 0.49	4.04 ± 0.91	7.79 ± 0.66	5.11 ± 0.71
2.	1.50	15.94 ± 0.82	8.52 ± 1.23	19.92 ± 3.46	9.12 ± 0.99
3.	2.25	27.27 ± 1.71	14.41 ± 2.44	33.54 ± 4.12	17.11 ± 1.23
4.	3.00	39.26 ± 1.13	22.52 ± 3.03	44.82 ± 5.71	29.44 ± 2.41
5.	3.75	53.59 ± 2.46	39.61 ± 4.12	59.66 ± 5.06	48.14 ± 3.11
6.	4.50	70.32 ± 3.86	47.65 ± 5.12	79.54 ± 6.49	56.84 ± 4.66
7.	5.25	88.78 ± 4.85	79.11 ± 6.13	96.96 ± 6.82	87.98 ± 4.86
8.	6.00	109.77 ± 7.62	85.84 ± 5.63	124.14 ± 7.01	96.77 ± 5.74
9.	6.75	132.92 ± 4.68	104.24 ± 7.82	152.24 ± 8.16	125.48 ± 6.45
10.	7.50	153.61 ± 7.66	134.41 ± 9.33	168.14 ± 8.99	156.78 ± 7.19
11.	8.25	177.27 ± 6.49	144.43 ± 10.12	201.42 ± 10.41	178.16 ± 8.16
12.	24.00	201.71 ± 7.16	159.41 ± 11.03	222.51 ± 10.77	188.44 ± 9.17

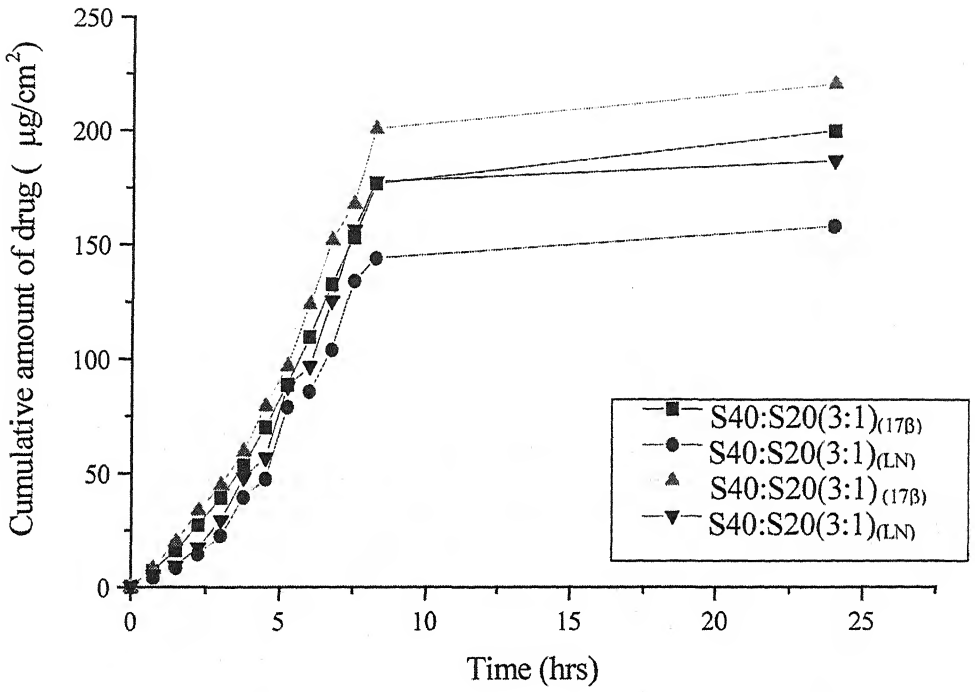


Figure 4.19: Permeation profile of Estradiol+Levonorgestrel from optimized proniosomal gel containing Spans, Span & Tween (Surfactant ratio) through rat skin; Span $_{40}$: Span $_{20}$ (S2) and Span$_{40}$: Tween $_{20}$ (T2).

Table 4.16: Steady state transdermal flux, permeability coefficient, regression value for transport of estradiol, ethinylestradiol & levonorgestrel

Formulation code	Cumulative drug release (µg)	Steady state transdermal flux (µg/cm^2.hr)	Permeability coefficient cm/hr	Regression value	Percentage release
S20:T20 (3:1)	393.88±9.91	14.50 ± 0.342	3.37 X 10^{-2}	0.80419	78.77
S20:T40 (3:1)	330.40±4.47	12.18 ± 0.311	3.13 X 10^{-2}	0.82492	66.08
S20:T60 (3:1)	299.66±4.66	11.04 ± 0.267	2.84 X 10^{-2}	0.81214	59.93
S20:T80 (3:1)	349.63±10.77	12.89 ± 0.346	3.32 X 10^{-2}	0.80615	69.92
S40:T20 (1:1)	323.92±9.77	11.94 ± 0.512	3.07 X 10^{-2}	0.80302	64.78
S40:T20 (2:1)	285.84±7.44	10.53 ± 0.402	2.71 X 10^{-2}	0.82529	57.16
S40:T20 (3:1)	274.82±6.88	10.13 ± 0.398	2.61 X 10^{-2}	0.85184	54.96
S40:T20 (4:1)	240.52±10.22	8.86 ± 0.668	2.28 X 10^{-2}	0.83842	48.10
S40:T20 (3:1)	278.84±8.13	10.28 ± 0.812	2.64 X 10^{-2}	0.82432	55.76
S40:T40 (3:1)	2.51.43±11.31	9.27 ± 0.712	2.38 X 10^{-2}	0.83817	50.28
S40:T60 (3:1)	240.54±9.32	8.86 ± 0.882	2.28 X 10^{-2}	0.83842	49.10
S40:T80 (3:1)	269.88±9.09	9.95 ± 0.744	2.56 X 10^{-2}	0.83619	53.97
S60:T20 (3:1)	258.42±9.98	9.52 ± 0.253	2.45 X 10^{-2}	0.83437	51.68
S60:T40 (3:1)	220.58±10.33	8.13 ± 0.598	2.09 X 10^{-2}	0.83535	44.11
S60:T60 (3:1)	206.02±8.41	7.59 ± 0.458	1.95 X 10^{-2}	0.83893	41.20
S60:T80 (3:1)	243.95±8.82	8.99 ± 0.789	2.31 X 10^{-2}	0.83057	48.79
S80:T20 (3:1)	364.12±8.39	11.08 ± 0.786	3.46 X 10^{-2}	0.82246	72.82
S80:T40 (3:1)	332.73±7.18	12.26 ± 0.346	3.16 X 10^{-2}	0.82531	66.54
S80:T60 (3:1)	300.42±6.77	11.07 ± 0.463	2.85 X 10^{-2}	0.82538	60.08
S80:T80 (3:1)	342.64±10.74	12.63 ± 0.289	3.25 X 10^{-2}	0.82468	68.52
S40:S20 (3:1)	224.05±8.75	8.26 ± 0.226	2.12 X 10^{-2}	0.81922	44.81
S40:T20 (1:1)	333.92±5.87	12.31 ± 0.338	3.17 X 10^{-2}	0.81783	66.78
S40:T20 (2:1)	295.84±8.70	10.90 ± 0.456	2.81 X 10^{-2}	0.84122	59.16
S40:T20 (3:1)	292.82±10.42	10.79 ± 0.678	2.72 X 10^{-2}	0.87565	58.56
S40:T20 (4:1)	250.52±9.81	9.23 ± 0.989	2.38 X 10^{-2}	0.86029	50.10
S40:S20 (3:1)	224.05±8.75	8.26 ± 0.226	2.12 X 10^{-2}	0.81922	44.81
S40:T20 (IPA)	340.40±9.83	12.55 ± 0.698	3.23 X 10^{-2}	0.83878	68.08
S40:T20 (AA)	319.34±10.60	11.77 ± 0.774	3.03 X 10^{-2}	0.85679	63.86
S40:T20 (P)	275.44±7.46	10.15 ± 0.623	2.61 X 10^{-2}	0.82697	55.08
S40:T20 (B)	256.56±6.99	9.46 ± 1.232	2.43 X 10^{-2}	0.82728	51.31

Cont'd....

Formulation code	Cumulative drug release (μg)	Steady state transdermal flux (μg/cm^2.hr)	Permeability coefficient cm/hr	Regression value	Percentage release
S40:T20 (3:1)	340.40±9.83	12.55 ± 0.698	3.23 X 10^{-2}	0.83878	68.08
S40:T20 (30 Sec)	350.87±8.63	12.93 ± 1.169	3.33 X 10^{-2}	0.82939	70.17
S40:T20 (60 Sec)	362.38±8.09	13.36 ± 0.995	3.44 X 10^{-2}	0.82865	72.47
S40:T20 (90 Sec)	440.32±6.48	16.23 ± 2.354	4.18 X 10^{-2}	0.80399	88.06
S40:T20 (120 Sec)	487.24±9.59	17.96 ± 3.578	4.63 X 10^{-2}	0.80741	97.44
S40:T20 (3:1)	340.40±9.83	12.55 ± 0.698	3.23 X 10^{-2}	0.83878	68.08
S40:T20 (EL)	354.05±5.83	13.05 ± 0.823	3.36 X 10^{-2}	0.81669	70.81
S40:T20 (SL)	421.16±8.91	15.52 ± 1.442	4.00 X 10^{-2}	0.81344	84.23
S40:T20 (DCP)	382.15±11.09	14.09 ± 1.124	3.63 X 10^{-2}	0.80944	76.43
S40:T20 (CHL)	348.12±7.36	12.83 ± 0.669	3.30 X 10^{-2}	0.82711	69.62
S40:T20 (17β)	368.18±6.06	13.57 ± 1.745	2.99 X 10^{-2}	0.81236	73.63
S40:T40 (17β)	298.51±5.94	10.99 ± 2.647	2.42 X 10^{-2}	0.83389	59.70
S40:T60 (17β)	263.75±6.00	9.72 ± 1.844	2.14 X 10^{-2}	0.84706	52.75
S40:T80 (17β)	319.84±10.77	11.99 ± 2.668	2.60 X 10^{-2}	0.82711	63.96
S40:T20 (LN)	348.21±8.93	12.43 ± 2.795	4.38 X 10^{-2}	0.82614	69.64
S40:T40 (LN)	248.14±7.99	9.14 ± 4.237	3.12 X 10^{-2}	0.84261	49.62
S40:T60 (LN)	242.18±9.08	8.92 ± 0.999	3.04 X 10^{-2}	0.85656	48.43
S40:T80 (LN)	258.15±7.19	9.51 ± 1.687	3.24 X 10^{-2}	0.83755	51.63
S40:S20 (EEE)	151.42±10.79	5.58 ± 4.689	1.43 X 10^{-2}	0.83621	30.28
S40:S20 (LN)	129.44±6.98	4.77 ± 6.798	1.62 X 10^{-2}	0.85715	25.88
S40:T20 (EEE)	165.99±7.73	6.12 ± 8.882	1.57 X 10^{-2}	0.85022	33.19
S40:T20 (LN)	158.72±10.42	5.85 ± 9.882	1.99 X 10^{-2}	0.86708	31.74
S40:S20 (17β)	201.71±7.16	7.43 ± 3.181	1.91 X 10^{-2}	0.82078	40.34
S40:S20 (LN)	159.41±11.03	5.87 ± 5.443	2.00 X 10^{-2}	0.79339	31.88
S40:T20 (17β)	222.51±10.77	8.20 ± 4.569	2.11 X 10^{-2}	0.81319	44.50
S40:T20 (LN)	188.44±9.17	6.94 ± 6.774	2.37 X 10^{-2}	0.79021	37.68

- Total amount of drug incorporated in the proniosomal gel was 500 μg each.
- Area of cellophane membrane & transdermal patch was – 1.13 cm^2
- Steady state transdermal flux =Amount of drug/time X Area of patch (Q/t X A)
- Permeability coefficient = Amount of drug/Saturated solubility of drug in receptor fluid
- Percentage release = $\frac{\text{Total amount of drug released}}{\text{Total amount of drug in formulation}} \times 100$

Fig. 4.20: Steady state transdermal flux value of different proniosomal gel formulations of estradiol, ethinylestradiol and levonorgestrel

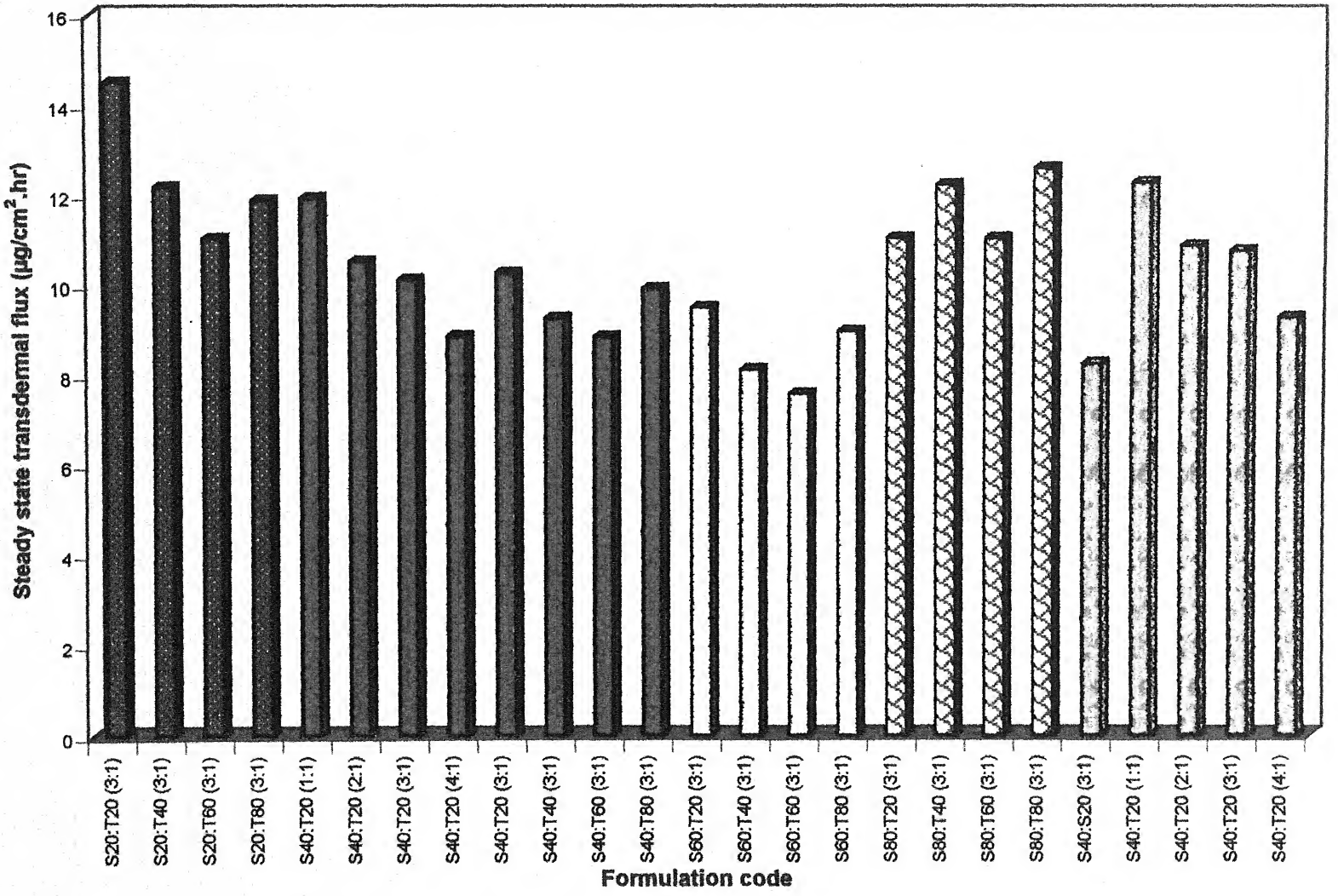
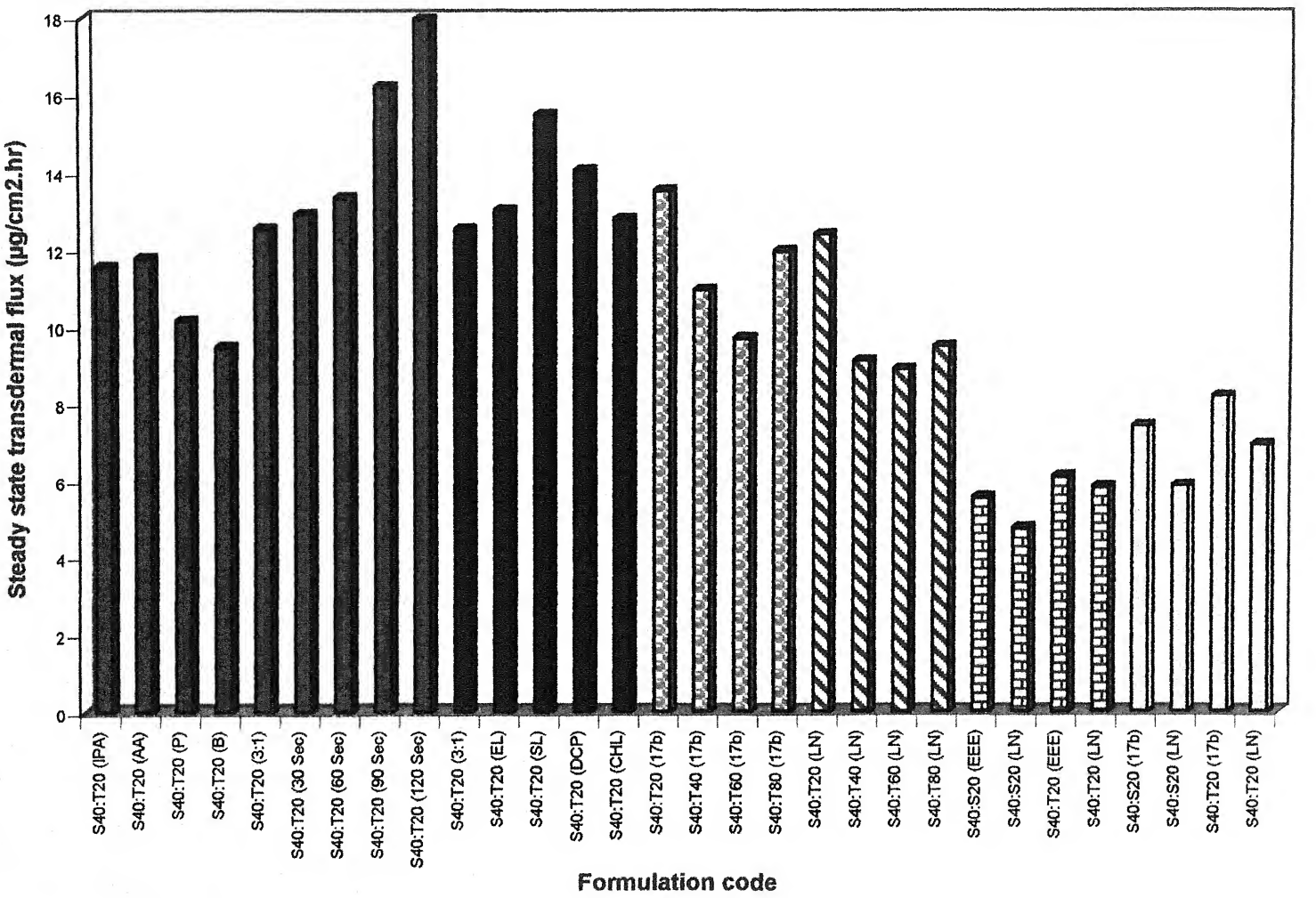


Fig. 4.20: Steady state transdermal flux value of different proniosomal gel formulations of estradiol, ethinylestradiol and levonorgestrel



RESULT AND DISCUSSION:

As discussed earlier, the preliminary formulation of proniosomal gels were designed using combinations of spans only. These formulations showed maximum release rate upto 60-65%. Therefore in order to optimize the drug release, the proniosomal gel formulations were designed by using the combinations of span with tweens in addition to span-span combination for achieving the desired transdermal flux of estradiol, ethinylestradiol and levonorgestrel.

Initially the proniosomal gel was developed with combinations of tweens with or without cholesterol as per method reported by Perrett *et al.*, (1990). But these combinations fail to produce proniosomal gel. Freely soluble non-ionic surfactants such as tween can form the micelles on hydration and in the presence of cholesterol above 30% concentration of the selected ratio of tweens, produced only liquid crystals either square shape or needle shape. This is because the vesicles cannot be formed by tweens only (Table 4). Hence Proniosomal gels were prepared by drop wise addition of water to lipid dissolved in alcohols by heating. The addition of water resulted in the precipitation of lipids in the form of hydrated bilayers. Similarly proniosomal gels were also prepared by addition of water to warmed solution of surfactants (Span + Tween) in alcohol. An initial proniosomal gel was prepared consisting of lipid or surfactant: alcohols: water in the ratio of 3:1(Span 40: Tween 20): alcohol (0.75 ml): Water (9 drops) to be used as a control formulation (Table 4a).

These proniosomal gels were chosen on the basis of (a) optical anisotropic structure observed under the cross polarizer equipped microscope and (b) formation of vesicles spontaneously upon hydration. More than 100 proniosomal formulation formulations using combinations of surfactants (Spans & Tweens) and alcohols were prepared in the same way, compositions of these gels are given in table 4 and 4a. Out of these, three formulations prepared using the combination of Span 40 and tween 20, Span 40, tween 20 and lecithine, and Span 40, tween 20 with cholesterol were selected for further studies because they showed the uniform vesicle size with good entrapment efficiency.

Proniosomes prepared with span 40 and tween 20 plus cholesterol produced compact niosomes appearance and semisolid consistency. In the case of proniosomes with egg lecithine and soya lecithin the mesophasic proniosomal gel stage was not arrested as reported for egg lecithin by Perret *et al.* (1991). While the optical anisotropic

compact niosome transition point was arrested. In order to optimize the drug loading in different proniosomal formulations those prepared using isopropyl alcohol were chosen on the basis of previous report by Paul *et al.* (1990) estradiol, ethinylestradiol and levonorgestrel have got minimum solubility in isopropyl alcohol as compared to other homologues alcohols Therefore, the release of these hormones was better from gels containing isopropyl alcohol than any other alcohol. This may due to the co-surfactant effect which has loosened the bilayer packing resulting in the release of flux value.

Mechanism of niosomes formation from span and tweens mixture of proniosomes is shown in photomicrographs 4.1-4.6. The results indicate that the process of dissolution may occur by progressive hydration of surfactant on the surface of proniosomes taking the form of niosomes as "budding off" from the outer surface of proniosomal gel. So long as the hydration took place, the formation of vesicles was continued. This may be due to the presence of tweens with span that could produce the hydration environment for more water absorption. It seems likely that the comparatively uniform size of niosomes formed under this relative static condition may result in the absence of shear force normally present during conventional hydration procedure. In this hydration method the bud like structures were seen at the outer surface. Middle layer was partially hydrated (optical anisotropic structure or partially formed vesicle) and inner core was unaffected. The observations are given in photomicrographs 4.1-4.6.

In case of topical drug delivery the size and size distribution study are very important parameter. The size and size distribution of vesicles was done by simple microscopic evaluation of niosome derived from proniosomal gel by the dilution of proniosomal gel is presented in the table 4.2. The size of vesicles formed was greater in case of hydration without agitation while it was less in case of hydration with agitation this may be attributed to breaking of vesicles due to shear force of agitation. The diameter of most niosomes appears to lay in the range of 1.52μm to 15.44 μm, little variation of size was seen between the batches of niosomes of given composition. The diameter of niosomes of span 40: span 20 was in the range of 8.84μm to 15.44μm, and ST22, 24, 26, 28 was 3.01μm to 3.82, ST42, 44, 46, 48 was 5.44μm to 6.63, ST62, 64, 66, 68 was 6.02 μm to 6.84 μm, and ST82, 84, 86, 88 was 4.12 μm to 5.22 μm, and size with different lipids was in range of 4.33μm to 7.99 μm. However the size of vesicle formed from proniosomal gel with cholesterol was uniform and very less. The polydispersity index

was always found very low showing that this method of niosome formation results in vesicles of highly uniform in size Table 4.2.

The calculated HLB value of prepared proniosomal gel given in the table 4.17.

Table 4.17: Reported and calculated HLB values of different Spans and Tweens combinations.

S. No.	**Reported HLB values**			
1	8.6	Span-20	Tween-20	16.7
2	6.7	Span-40	Tween-40	15.6
3	4.7	Span-60	Tween-60	14.9
4	4.3	Span-80	Tween-80	15.0
	Calculated HLB value			
5	7.65	Span -20: Span 40(1:1)	Span-80+Tween 60(3:1)	4.4
6	7.96	Span -20: Span 40(2:1)	Span-80+Tween 40(3:1)	4.9
7	8.12	Span -20: Span 40(3:1)	Span-20+Tween 60(3:1)	7.62
8	8.22	Span -20: Span 40(4:1)	Span-20+Tween 80(3:1)	7.52
9	7.65	Span -40: Span 20(1:1)	Span-40+ Tween 20 (1:1)	11.6
10	7.33	Span -40: Span 20 (2:1)	Span-40+ Tween 20 (2:1)	9.96
11	7.17	Span -40: Span 20 (3:1)	Span-40+ Tween 20 (3:1)	9.15
12	7.08	Span -40: Span 20 (4:1)	Span-40+ Tween 20 (4:1)	8.48
13	10.57	Span-20+Tween-20 (3:1)	Span-60+Tween-20 (3:1)	7.65
14	10.35	Span-20+Tween-40 (3:1)	Span-60+Tween-40 (3:1)	7.425
15	10.17	Span-20+Tween-60 (3:1)	Span-60+Tween-60 (3:1)	7.25
16	10.20	Span-20+Tween-80 (3:1)	Span-60+Tween-80 (3:1)	7.275
17	9.15	Span-40+Tween-20 (3:1)	Span-80+Tween-20 (3:1)	7.35
18	8.925	Span-40+Tween-40 (3:1)	Span-80+Tween-40 (3:1)	7.125
19	8.75	Span-40+Tween-60 (3:1)	Span-80+Tween-60 (3:1)	6.95
20	8.775	Span-40+Tween-80 (3:1)	Span-80+Tween-80 (3:1)	6.975

Spans span combinations (HLB value 7.08 to 7.65) the vesicle size was more (8.44μm-15.44μm) in comparison to formulation prepared using span tween combination (HLB value 8.75 to 9.15). But span 40 + tween 20 produced medium size (5.44 μm to 6.63 μm) uniform vesicles. Therefore, span 40 with tween 20 were considered to be optimum formulation because incorporation of tween had reduced the size of vesicles. Table 4.17.

The phase transition temperature of span 40 is higher in comparison to span 20, which affects the permeability of bilayers. Therefore, as the proportion of span 40 changed the permeability also changed. Thus the size was found to decrease with increasing HLB value of mixture but drug entrapment efficiency also decreased in the same order.

In the case of different combinations of span and tween the size of vesicle was decreased with increasing HLB value. But the entrapment efficiency was more in case of span- span combination while it was less in case of combination of span with tween.

These results indicates that the drug entrapment (estradiol, ethinylestradiol and levonorgestrel) was more in the case of surfactants combination of low HLB value (hydrophobic) and decreased as the HLB value of surfactant combination was increased. This may be accounted for the hydrophobic nature of drug of drug.

The entrapment efficiency was higher in case of spans combination and overall entrapment efficiency was in decreasing order of

SS42 > ST42 with lipid > ST22 > ST42 >ST 62 > ST82, where S= span and T=tweens. Table 4.2.

The microscopic study revealed that the vesicle size was in decreasing order of

SS42 > ST42 with lipid > ST62 > ST82 >ST 42 > ST22.

This again may be attributed to hydrophilic nature of tweens forming small vesicles.

From different composition of lipids, the vesicle size in niosomes were found to be in the increasing order of ST42CHL < ST42DCP < ST42 EL < ST42 SL. This may be was attributed to the varying factors like change in solubility, effect of bilayer integrity and their intrinsic composition. In the case of egg and soya lecithin a proper conclusion could not be made. The entrapment efficiency of niosomes prepared using lipids was found in the increasing order of ST42EL < ST42SL < ST42 DCP < ST42 CHL. This order reflected the rigidization effects of various additive lipids. The lipid causing the highest rigidization of bilayer i.e. cholesterol shows highest entrapment efficiency .the study of vesicle size and percent (%) drug entrapment of different proniosomal formulations revealed that in case of large vesicle percent (%) of drug entrapment was more as compared to small vesicles.

Vesicles formed from different alcohols were of different sizes. They followed the increasing order in size as Isopropyl alcohol < Absolute alcohol < Propanol < Butanol. Vesicles with isopropyl alcohol results in smallest size may be due to branched chain present in it and larger size was found with other homologues alcohols. These results are in accordance with findings report by Ishii *et al.* (1995).

It has been accepted that on administration to biological system the *in-vitro* release rate or permeation profile provides valuable information about the products behavior in-vivo. In the present study 10 ml of 40% PEG 200 solution was used as receptor fluid for the *in vitro* drug release. The 40% PEG 200 solution was used to maintain the sink condition on the basis of solubility consideration of estradiol, ethinylestradiol, and levonorgestrel. In earlier reports also Brain *et al.* (1993), have used 40% PEG 200 to maintain good sink condition for estradiol. Lies *et al.* (1991) have also reported that only the elution fluid containing 40% PEG 400 v/v was able to maintain good sink condition for release rate of progesterone from matrix type drug delivery system. Catz and Friend (1990) have reported that 40% PEG 400 in saline as the receptor fluid increased slightly the steady state flux of levonorgestrel relative to saline as the receptor solution.

The release rate curve of ethinylestradiol in 40% PEG 200 from different formulations through cellophane membrane is shown in Figures 4.7-4.15. The cumulative amounts released from different formulations in 24 hrs are summarized in table 4.3-4.11.

In vitro release profiles were studied in triplicate and standard deviation, transdermal flux, permeability coefficient, release rate, and percentage release were calculated from the data and reported in the table 4.16. No lag phase could be detected because the minimum sampling time was 45 minutes and drug permeation from proniosomal formulation through cellophane membrane was found to follow zero order release kinetics. Data of permeation profile of three drugs estradiol, ethinylestradiol, and levonorgestrel through cellophane membrane were linearly regressed and fitted in the straight line to get slope value.

The correlation coefficient between the skin permeation from all formulation tested was calculated. The r-value obtained was close to one showing zero order release profile for all the proniosomal formulations. The values were 0.79021 to 0.99931

i.e. nearly one showing linear release profile. The drug release from formulations was in following order for mixture of spans and tweens.

ST22>ST82>ST42>ST62. The reason for the increased drug release may be the increase in HLB value with the addition of tween 20, 40, 60 and 80. From all these formulations of span 20 with tween 20 and 80 were found to be in the liquid state while composition of span 40 and 60 with span 20 were in ordered gel state (Figure 4.7-4.11).

In order to see the effect of alcohols formulation ST42 was prepared with different alcohols and applied to treat cellophane membrane. The apparent steady state transdermal flux was highest for the formulation containing isopropyl alcohol. Inclusion of absolute alcohol, and propanol, and butanol into the formulation also led to the enhanced drug release but the effect was less than Isopropyl alcohols. This may be due to the branched chain structure of isopropyl alcohol, which acts as a co-surfactant and might have reduced the bilayer packing with resultant increase in the flux value. The presence of tweens that can easily equilibrate with the alcohols for hydration also enhanced the transdermal flux value. The effect of alcohols on the release rate of drugs from plain formulation prepared with different alcohols was observed in the following decreasing order (Figure 4.13).

(ST42) Isopropyl alcohol > (ST42) Absolute alcohol>(ST42) Propanol >(ST42) Butanol.

Effect of sonication time on the selected formulation the flux was highest after 120 seconds of ultrasonication. The flux was found to increase with the sonication time. This may be due to the membrane disruption reducing vesicle size and energy mediated mixing of vesicle compositions and skin lipids (Figure 4.14).

In case of proniosomes prepared using mixture of spans the release rate was found to be in increasing order if the amount of span20 was increased SS24 (ratio 1:1)<SS24 (ratio 2:1)<SS24(ratio3:1)<SS24(ratio 4:1) and on increasing the ratio of span 40 the release rate was found in the order of SS42(ratio 1:1)>SS42(ratio 2:1)>SS42(ratio 3:1)>SS42 (ratio 4:1). Among all the combination of spans and span & tweens the release rate was found to be in the following decreasing order in case of T20, 40, 60, 80 with span 20 > T20, 40, 60, 80 with span 80 > T20, 40, 60, 80 with span 40 > T20, 40, 60, 80 with span 60. This was in accordance with the phase transition temperature of the surfactant mixture. The effect of different spans on the release rate showed that the

highest flux value was in the case of span 20 and span 80 because span 20 and 80 has low phase transition temperature. The lower flux value was obtained in the case of span 40 and span 60. Further the slow release may be due to the reduction in surface free energy that caused the formation of larger size vesicles resulting smaller surface area exposed to receptor medium and membrane or skin. The low phase transition temperature of span 20 combinations allowed the faster release than span 80 combinations

All the formulations containing spans plus tweens combinations released significantly less estradiol, ethinylestradiol than phospholipid formulation. The release rate was in following decreasing order of

ST42SL >ST 42DCP > ST42 EL > STCHL>ST24.

In the case of formulation with varying composition the drug release was maximum in soya lecithin and dicetyl phosphate formulation showed intermediate release because its vesicle are charged which is responsible for increasing the curvature and decreasing the size of vesicle with the increase in surface area. Both the commercial soya lecithin and egg lecithin were effective penetration enhancers. The drug Release rate was more in soya lecithin formulation than egg lecithin. Higher skin permeability may be due to an increase in partition coefficient between vehicle and skin or direct effect of lecithin on the skin thereby reducing the skin resistance to permeation of the drugs. Both soya and egg lecithine contain phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol and poly-unstarated acids including lenoleic acid and lenolenic acid. These polyunsaturated fatty acids are relatively more in soya lecithin that may be the reason for faster release rate than egg lecithin. The cholesterol increases the rigidity of bilayers hence slowest release rate was observed (Figure 4.15).

On the basis of these release rate studies formulation ST42 (T1) was found to exhibit optimum drug release and permeation through the membrane. The formulation containing estradiol, ethinylestradiol, and levonorgestrel were prepared separately with or without tweens and lipids and evaluated for drug release studies. The effect of soya lecithin, egg lecithin, dicetylphosphate and cholesterol on the drug release rate from the formulations was also studied. After optimization of these formulations containing single drug, two formulations of span: span combination were prepared containing drug combination i.e. estradiol with levonorgestrel (1mg+1mg) and ethinylestradiol with levonorgestrel (1mg+1mg) and coded as S-1 and S-2. The formulations of span 40 with

tween 20 were prepared containing same drug combinations and coded as T-1 and T-2. The release rate was found higher in the case of T-1 and T-2. This may be due to the higher transdermal flux of span and tweens formulation in comparison of spans combination only. This may be due to the better hydration of proniosomal gel in presence of tween (Figure 4.18-4.19).

Chapter-5

STABILITY STUDIES

The stability of any pharmaceutical product is the capability of the delivery system in the prescribed formulation to remain within defined or prescribed limits for a predetermined period of time. There is no established protocol for either accelerated or long-term stability studies for the proniosomal formulations though different scientists study many of them. However, the traditional guidelines that are generally observed for pharmaceutical dosage forms are followed while adopting the environmental conditions of the Institutional set up the stability studies are broadly covered under two main sections (Vyas and Khar, 2002). First, the stability study in-vitro, which covers the stability aspects prior to administration of the formulation and with regard to the stability of constitutive non-ionic surfactant. Second, the stability in-vivo, which covers the stability aspects once the formulation, is administered via various routes to the biologic fluids.

The stability of alkyglycoside vesicle was compared with phosphatidylcholine based liposomes vesicles both containing lipid: DCP: Cholesterol 4:1:2, it was observed that the phosphatidylcholine based vesicle disintegrated in-vitro after 22 weeks, while niosomes prepared using alkyl glycoside endured at least for 25 weeks (Baillie *et al.*, 1985).

One must treat liposomel/ niosomal vesicular drug delivery systems in the same way as more traditional pharmaceutical dosage forms are treated with respect to the established and clearly defined protocols for their characterization, manufacture, stability testing and their efficacy.

Procedure: The proniosomal formulations S1, T1 and T_{CH} were prepared by the coacervation phase separation method and stored in glass vials with cap of 5.0 ml capacity and stored at

Freeze temperature - (4°C)

Moderate room temperature - (25±2°C)

Higher temperature - (40±2°C)

After storage in the glass vials, allow these vials were kept at different temperature conditions for 4 weeks, 8 weeks, 12 weeks and 16 weeks, they were observed visually under optical microscope for the change in their consistency, optical anisotropic structure and appearance of drug crystals upon storage. The observations are recorded in the table 5.1.

Table 5.1: Stability observations of selected proniosomal formulations for consistency, optical anisotropic structure and crystals appearance

S. No	Formulation	4 weeks,			8 weeks			12 weeks			16 weeks		
Temperature		4°C	27°C	46°C	4°C	27°C	46°C	4°C	27°C	46°C	4°C	27°C	46°C
1	Consistency of												
	S1	NC	NC	NC	NC	NC	NC	CH	CH	CH	CH	CH	CH
	T1	NC	NC	NC	NC	NC	NC	CH	CH	CH	CH	CH	CH
	T_{CH}	NC	NC	NC	NC	NC	NC	NC	NC	NC	CH	CH	CH
2	Optical												
	anisotropic	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
	structure	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
	S1	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
	T1												
	T_{CH}												
3	Appearance of												
	drug crystals												
	S1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	A
	T1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	A	A
	T_{CH}	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

NC = Not changed **CH** = Changed **NA** = Not appeared **A** = Appeared

The proniosomal gels were stored for 4, 8, 12 and 16 weeks and converted into niosomes after hydration with agitation or without agitation and were characterized for size and size distribution. About 100 mg proniosomal gels of SS 42 (S1) and ST 42 (T1) were hydrated in small test tube by adding 10 ml of saline solution (0.154 M NaCl) and manually shaken for 10-15 minutes. The dispersion was observed under optical microscope at 1000 × magnification. The observations are given table 5.2, 5.3 and 5.4.

RESULT AND DISCUSSION:

Proniosomes were stored at room temperature 4°C, 25±2°C and 40±2°C in clean glass vials, prior to hydration, appearance of crystals and size were determined by observing under microscope. Drug crystals were not seen after 12 weeks in case of formulation of SS 42 (S1) and ST 42 (T1) while in case of ST42 with cholesterol (T_{CH}) crystals were not observed even after 16 weeks. The consistency of proniosomal gel SS42 (S1) was increased after 12 weeks storage and in case of ST42 CHL ($T1_{CH}$) consistency

was increased after 16 weeks. There was no significant difference in the optical anisotropic structure of proniosomal gel formulation even after 16 weeks storage time (Table 5.1).

The polydispersity analysis of formulation containing SS42 (S1), ST42 (T1) and ST42CHL (T_{CH}) was performed and niosomes were counted by using simple microscope at 1000X magnification. Size analysis of niosomes formed after hydration of proniosomes stored for 4, 8, 12 and 16 weeks at 4°C, 25±2°C and 40±2°C are also performed and the results are summarized in table 5.2, 5.3 and 5.4.

Observation of table 5.2, 5.3 and 5.4 indicates that only minor changes were found in niosomes derived from proniosomes. These changes principally affected formulation after 12 weeks of storage at room temperature and after 16 weeks at 4°C, 27±2°C and 40±2°C.

Table 5.2: Characterization of niosomes formed from proniosomal gels after storage at freeze temperature (4°C)

S. No	Time in weeks	Formulation Selected for study							
		S40:S20 (3:1)-S1 Size in μm				S40:T20 (3:1)-T1 Size in μm			
		Without agitation		With agitation		Without agitation		With agitation	
1.	4	8.68±0.377	0.043	5.47±0.257	0.046	7.35±0.361	0.049	2.43±0.124	0.092
		7.08±0.353	0.064	4.28±0.260	0.060	5.57±0.258	0.046	1.87±0.061	0.032
2.	8	10.61±0.405	0.047	7.23±0.359	0.077	7.95±0.352	0.056	2.55±0.129	0.050
		7.82±0.345	0.056	5.81±0.261	0.044	6.46±0.339	0.052	2.02±0.119	0.058
3.	12	11.35±0.450	0.039	9.15±0.399	0.043	8.69±0.362	0.041	3.00±0.121	0.040
		8.44±0.352	0.065	5.71±0.248	0.043	6.96±0.358	0.051	2.16±0.128	0.059
4.	16	12.12±0.522	0.059	10.44±0.386	0.036	9.46±0.419	0.044	4.44±0.271	0.061
		9.16±0.401	0.043	6.62±0.349	0.052	7.96±0.382	0.047	3.84±0.136	0.034

Table 5.3: Characterization of niosomes formed from proniosomal gels after storage at Room temperature (25±2°C)

S. No	Time in weeks	Formulation Selected for study							
		S40:S20 (3:1)-S1 Size in μm				S40:T20 (3:1)-T1 Size in μm			
		Without agitation		With agitation		Without agitation		With agitation	
1	4	10.41±0.391	0.037	7.34±0.365	0.049	5.90±0.318	0.053	2.82±0.132	0.046
		5.50±0.302	0.054	4.13±0.61	0.063	4.14±0.249	0.060	2.84±0.142	0.050
2	8	10.62±0.406	0.038	7.87±0.349	0.044	6.98±0.349	0.050	4.41±0.271	0.061
		6.19±0.332	0.053	4.81±0.282	0.058	5.38±0.301	0.055	4.01±0.256	0.063
3	12	11.07±0.411	0.037	8.00±0.366	0.045	8.21±0.344	0.041	5.57±0.343	0.061
		6.43±0.341	0.053	5.08±0.219	0.043	5.71±0.362	0.063	4.20±0.259	0.061
4	16	13.49±0.704	0.052	9.37±0.422	0.045	9.50±0.424	0.044	6.03±0.342	0.056
		6.63±0.351	0.051	5.13±0.288	0.056	5.91±0.372	0.062	5.12±0.291	0.056

Table 5.4: Characterization of niosomes formed from proniosomal gels after storage at higher temperature (40±2°C)

S. No	Time in weeks	Formulation Selected for study							
		S40:S20 (3:1)-S1 Size in μm				S40:T20 (3:1)-T1 Size in μm			
		Without agitation		With agitation		Without agitation		With agitation	
1	4	9.07±0.399	0.043	4.17±0.258	0.054	5.12±0.291	0.056	3.75±0.129	0.034
		4.14±0.258	0.062	3.99±0.139	0.059	4.17±0.253	0.063	2.420.122	0.050
2	8	9.54±0.419	0.045	4.56±0.275	0.062	5.25±0.299	0.056	3.92±0.132	0.060
		4.18±0.261	0.064	4.05±0.247	0.085	4.30±0.231	0.076	2.43±0.124	0.092
3	12	10.30±0.381	0.036	4.86±0.286	0.079	6.01±0.341	0.056	4.32±0.269	0.062
		4.81±0.291	0.082	4.33±0.270	0.062	4.33±0.270	0.085	2.80±0.139	0.049
4	16	10.81±0.415	0.047	5.13±0.295	0.076	7.34±0.371	0.051	5.02±0.281	0.055
		5.08±0.288	0.076	4.45±0.272	0.061	4.00±0.255	0.063	2.64±0.134	0.050

The consistency of gel formulation was increased due to the slow evaporation of alcohol. Another reason for increase in consistency may be due to the molecular interaction of polar head of surfactants with solvents and exchange of solvent into the bilayer. This solvent exchange mechanism does not affect optical anisotropic structure rather it resulted in complete bilayer formation due to the saturation of lipid polar head, which increased bilayer distance and consistency.

Result of polydispersity index during the size and size distribution study have shown increase in vesicle size and decrease in polydispersity index, which indicated the complete swelling of bilayers. Proniosomes hydration temperature (provided it is above the phase transition temperature of the components phospholipids) has been shown to have little or no effect on niosomes size. In cholesterol formulation, no appreciable difference in size and size distribution was observed during storage time period. While this study of proniosomes stability has not been exhaustive, the result suggests that proniosomes may offer a stable system for niosomes production. Thus, the increase in consistency was seen after 12 weeks in the case of proniosomal gel ST 42 (T1) and after 16 weeks in ST42 CHL (T_{CH}). There were no change in the optical anisotropic proniosomal structure at the reported temperature, and crystals also were not seen in both formulations during storage time at the same temperature.

Chapter-6

IN VIVO EVALUATION

In vivo evaluation of selected products was performed in female rats for the following parameters:

(a) Endometrial Effects

(b) Vaginal cornification

(c) Vaginal thickness

(d) Effect of estradiol, ethinylestradiol, and levonorgestrel and their combinations on ovulation point

(e) Growth response of uterus /ovary with estradiol, ethinylestradiol, and levonorgestrel

(f) Growth response of uterus/ovary with estradiol and levonorgestrel combination

(g) Growth response of uterus/ovary with estradiol & levonorgestrel combination

(h) Effect of estradiol, ethinylestradiol, and levonorgestrel and their combinations on lipid profile of female rats.

Female (Sprange-dawley Strain) rats weighing 125-150 gm were procured from animal house of Defense Research and Development Establishment, Gwalior. These animals were kept for one week to become familiar with the new environment at proper care and feeding to them. The rats were given standard diet with drinking water ad libitum. A temperature of 20-22°C was maintained with a relative humidity of 45-65% and 12:12 h light dark cycle. Animals were acclimatized for at least 7 days before each experiment and were observed daily for clinical signs of illness. Thirty-six female rats were taken each weighing 125-150 gm and divided into six groups. The hairs on the right dorsal side (neck side) of all the animals of six groups were cut by using scissors. After this depilatory (Anne French) was applied in this area for 15-20 minutes to remove all the hairs and cleaned the skin properly. 5.0 ml of 0.1 % copper acetate solution was injected intraperitonially to synchronize all the thirty-six female rats. After 24 hrs, the selected formulations in the form of transdermal patches having the diameter of 1.20 cm (Area-1.13 cm^2) were applied on the depilated skin of the animals. The animals of group-I were applied with blank patch containing only surfactants, which were treated as control. The proniosomal formulation ($T1_{LN}$) levonorgestrel was applied on II group, formulation-containing ($T1_{17\beta}$) containing estradiol on group III, formulation containing ($T1_{EEE}$) ethinylestradiol on group IV, formulation containing ($T1_{17\beta+LN}$) estradiol and levonorgestrel on group V, formulation containing ($T1_{EEE+LN}$) ethinylestradiol and levonorgestrel on group VI.

All these patches were applied at clean saved area and then fixed by using dressing tape (Leukoplast Beierdorf, India Ltd., Goa). The rats of each group were kept in separate cages, conditioned and maintained on laboratory rats feed with water ad libitum.

BIOLOGICAL ASSAY:

The rats of all groups under transdermal patch treatment were kept undisturbed and after one day, third day and seven day, rats of all the groups were sacrificed by excessive chloroform inhalation. The animal was dissected uterus, the ovaries were cut, and the fatty material was removed. Uterus horns and ovaries were weighed after dehydration with absolute alcohol on an electronic digital pan balance (Sisco popular, Varanasi, India). The ovaries were examined visually for the presence of ovulation point or bleeding points. Just after this, the organs were kept in xylene over night to make the tissue hard. After 24 hrs, tissue were washed with xylene, cleaned and blocks were made by embedding the tissue in matured wax. After forming these blocks, sections were out using a Microtome of 5.0 μm, thickness and the frozen section was mounted on a slide after heat treatment and stained with Ehrlich's hematoxylene and eosin and examined for histopathological changes in the uterine horns and ovaries. The estrogenic activity of estradiol and ethinylestradiol delivered from the transdermal patch and combined formulation in same manner was evaluated by the method reported by Hebborn (1971).

ESTROGENIC ACTIVITY

Uterine weight assay:

Only immature rats were used for this study. The transdermal patches were applied on the neck portion of rats, and sacrificed after one day, third day, seventh day after application of transdermal patch. The uteri were separated from the vagina by cutting through the cervix, the surrounded tissue was stripped off and the uterotubal junction severed. The uteri were then pressed before the blotting paper to express out the intrauterine fluid and weighed immediately in the wet state and also after dehydration with absolute alcohol.

Vaginal opening:

Normal female rat at maturation are observed to go through stages of vaginal opening. The patch was applied on the neck of 21 days old rats and time of complete vaginal opening and its diameter was observed as a sign of estrogenic activity.

Vaginal cornification:

Holding the animal ventral side up, a drop of water was inserted into the vagina with a pasture pipette or by syringe. Then the vaginal fluid was withdrawn after five minutes and transferred to microscopic slide and allowed to dry. It is very simple to place the smears obtained, on following days from the same animal, on the same slide so that comparison of the smears may be more readily be made. Each slide was marked with a wax pencil to identify the various smears, by placing the slide in absolute alcohol for 5 sec, allowing it to dry and staining it with a 5% aqueous methylene blue solution for 10 minutes. The excess stain was washed off with tap water and the slide was dried and observed using a microscope of low magnification. The slides were evaluated and categorized in following classes:

(a) O= Diestrous smears, mainly leukocytes, few epithelial cells

(b) X= Mixture of leukocytes and epithelial cells.

(c) Y= Progesterous smears, Nucleated or nucleated plus cornified cells, no leukocytes.

(d) I= Estrous smears cornified cell only. (Photomicrographs 6.8-6.12).

RESULT AND DISCUSSION:

The method reported by Hebborn was used to perform *in-vivo* study of transdermal patches containing proniosomal gels of estradiol, ethinylestradiol and levonorgestrel in albino rats of 23-24 days old; weighing 125-150 gm. For estrogenic activity, the proniosomal formulation ($T1_{17\beta}$) and $T1_{(EEE)}$ was selected and patch having an area 1.13 cm^2 containing formulation equivalent to 1 mg drug selected on the basis of *in vitro* drug release rate was applied to the animals in order to obtain the desired flux. The estrogenic activity was evaluated on the basis of following parameter:

(a) Vaginal Opening

(b) Smear Count

(c) Uterine weight

Vaginal Opening was found to be open fully in case of proniosomal gel formulation containing estradiol and little less in the case of proniosomal gel formulation containing ethinylestradiol. It was observed that 100% vagina was opened in comparison to initial vaginal condition of control group.

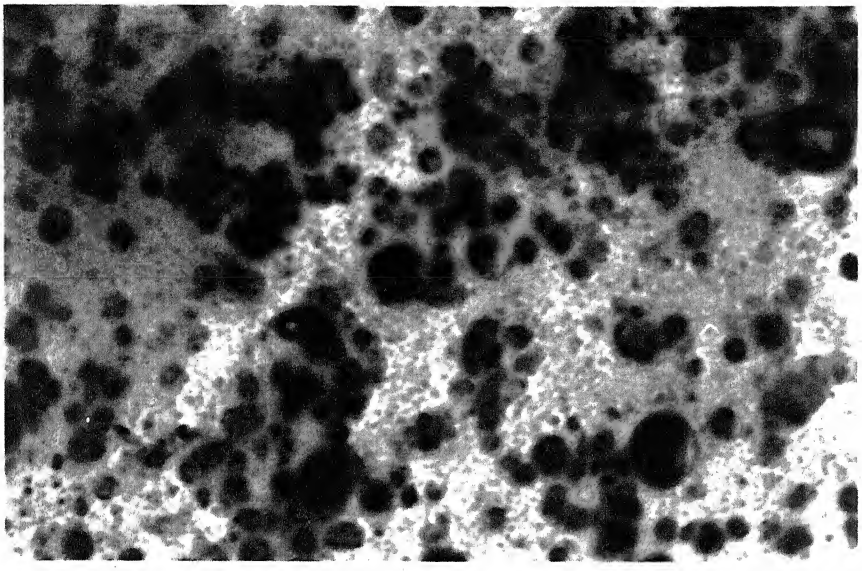


Figure-6.1: Photomicrographs showing smears of vaginal fluid of control rat after first day containing leukocytes with some granulated cell and epithelium cell

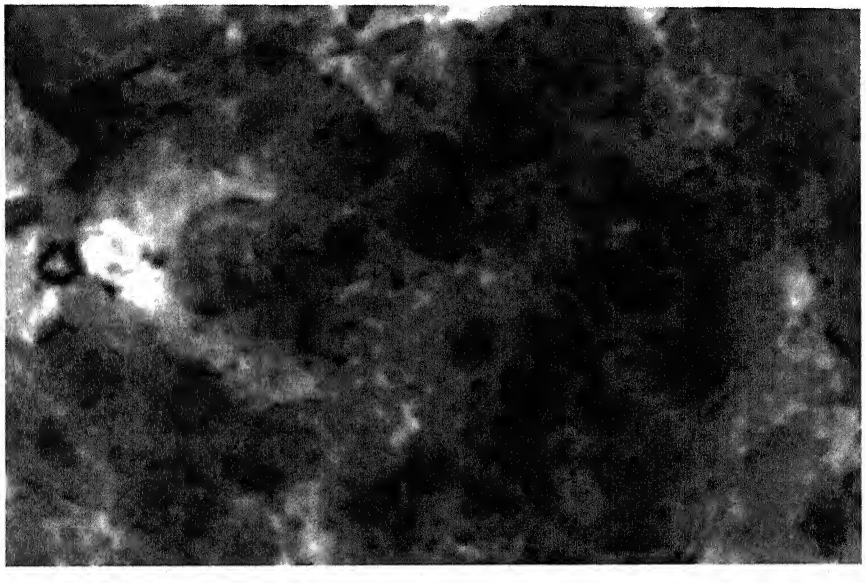


Figure-6.2: Photomicrographs showing smears of vaginal fluid of control rat after fourth day containing leukocytes with some granulated cell and epithelium cell

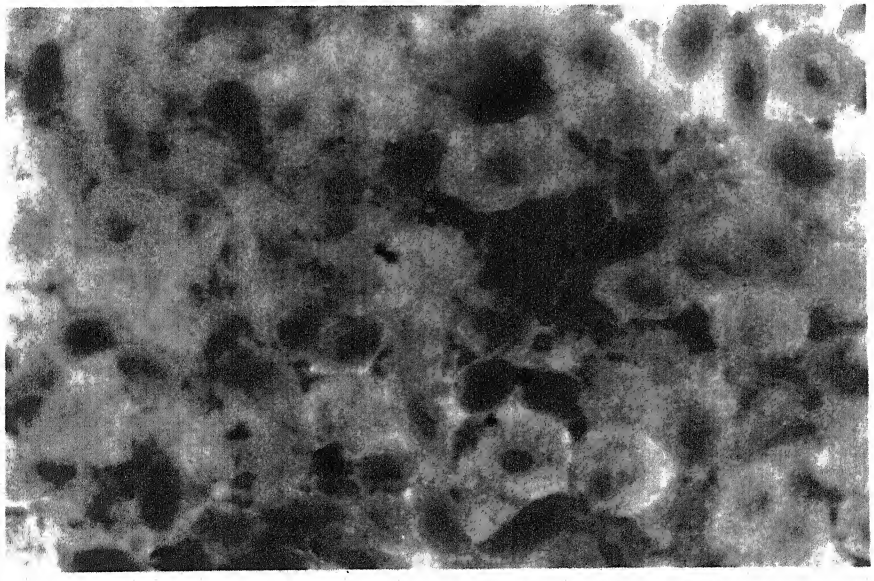


Figure-6.3: Photomicrographs showing smears of vaginal fluid of control rat after seventh day containing leukocytes with some granulated cell and epithelium cell

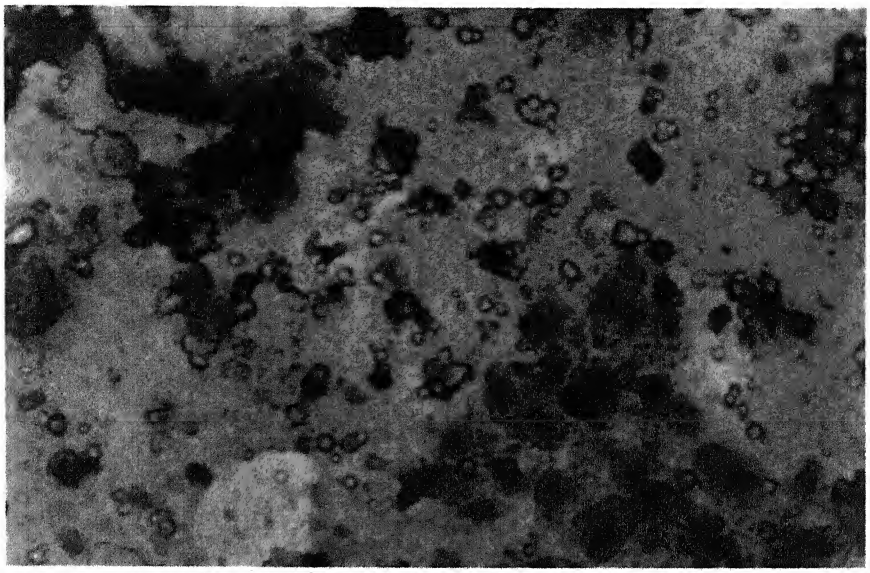


Figure-6.4: Photomicrographs showing smears of (viscous) vaginal fluid containing nucleated cells only in rat treated with proniosomal formulation containing levonorgestrel after first day

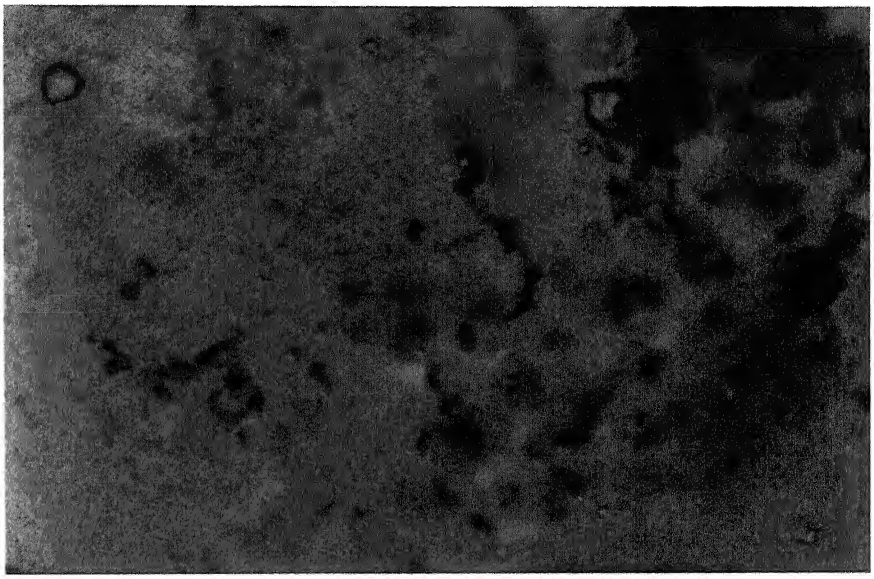


Figure-6.5: Photomicrographs showing smears of (viscous) vaginal fluid containing nucleated cells plus partially cornified cell in rat treated with proniosomal formulation containing levonorgestrel after fourth day

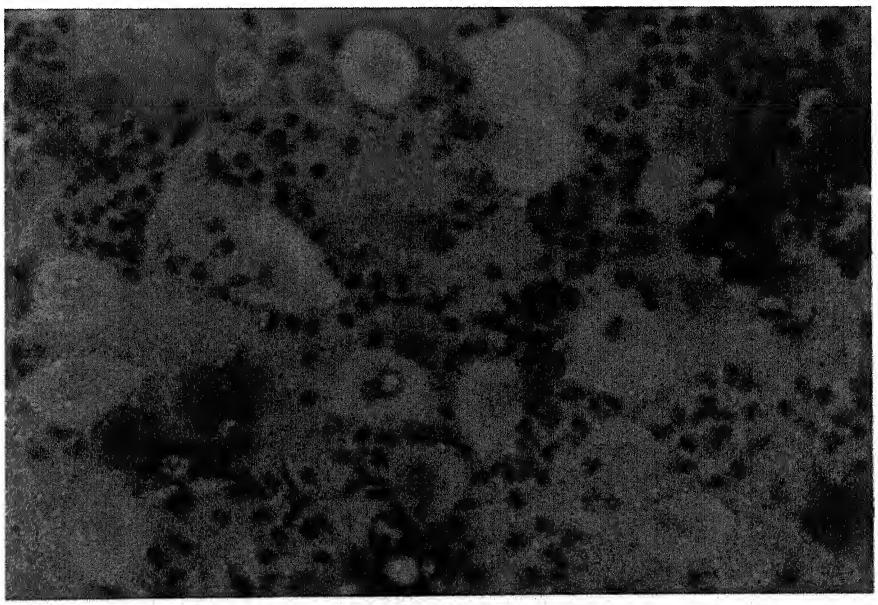


Figure-6.6: Photomicrographs showing smears of (viscous) vaginal fluid containing nucleated cell plus cornified cell, no leukocytes in rat treated with proniosomal formulation containing levonorgestrel after seventh day

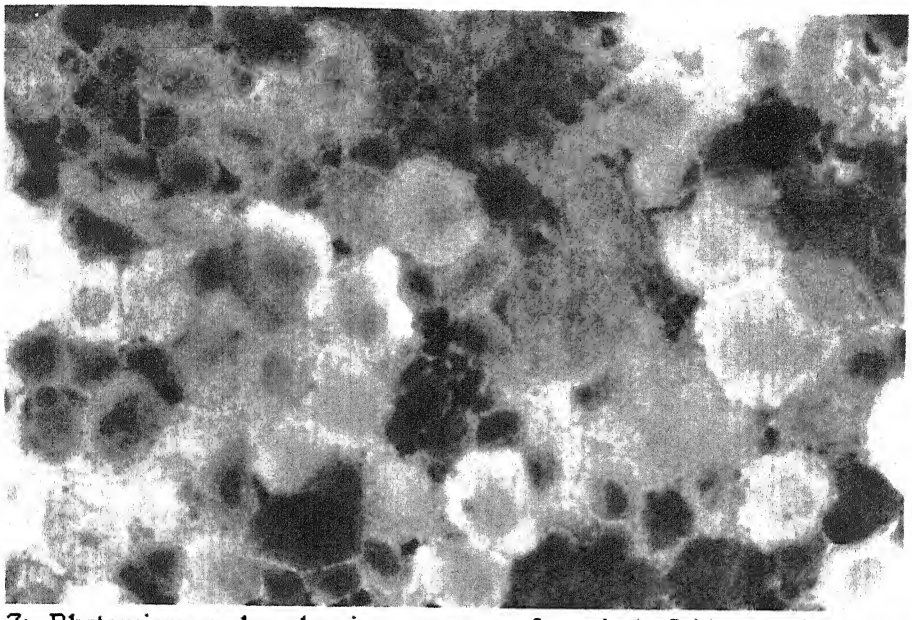


Figure-6.7: Photomicrographs showing smears of vaginal fluid containing leukocytes and epithelial cells in rat treated with proniosomal formulation containing estradiol after first day

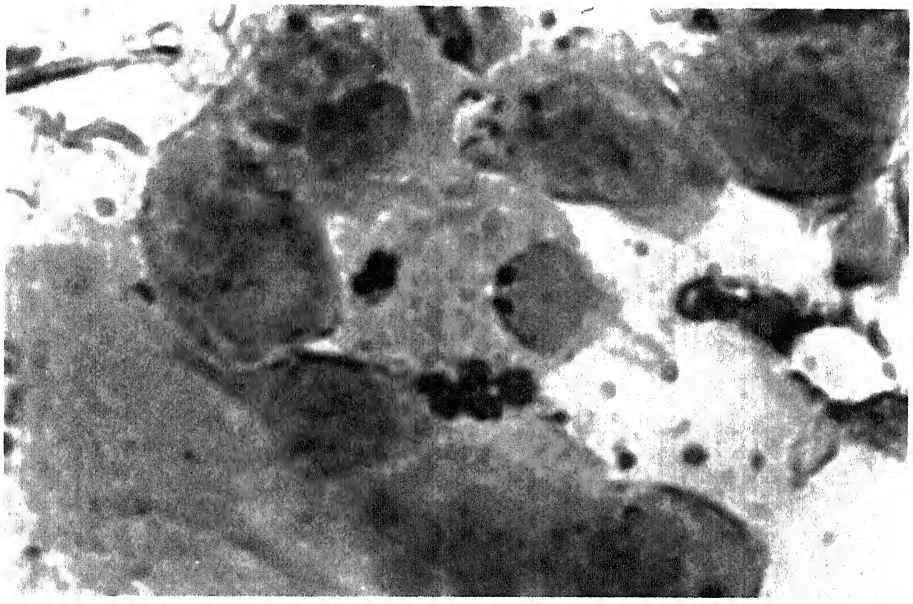


Figure-6.8: Photomicrographs showing smears of vaginal fluid containing leukocytes and epithelial cells and partially cornified cells in rat treated with proniosomal formulation containing estradiol after fourth day

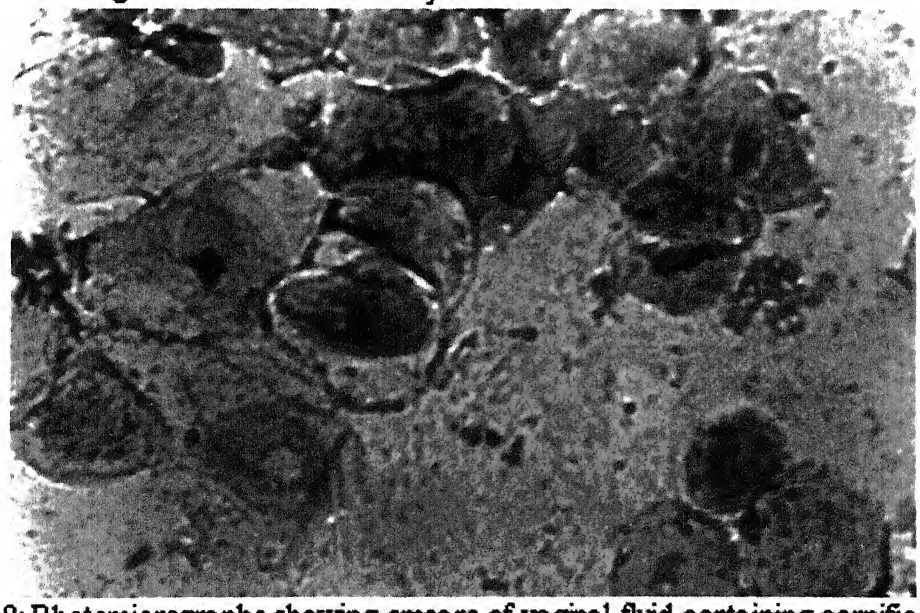


Figure-6.9: Photomicrographs showing smears of vaginal fluid containing cornified cells, no leukocytes in rat treated with proniosomal formulation containing estradiol after seventh day

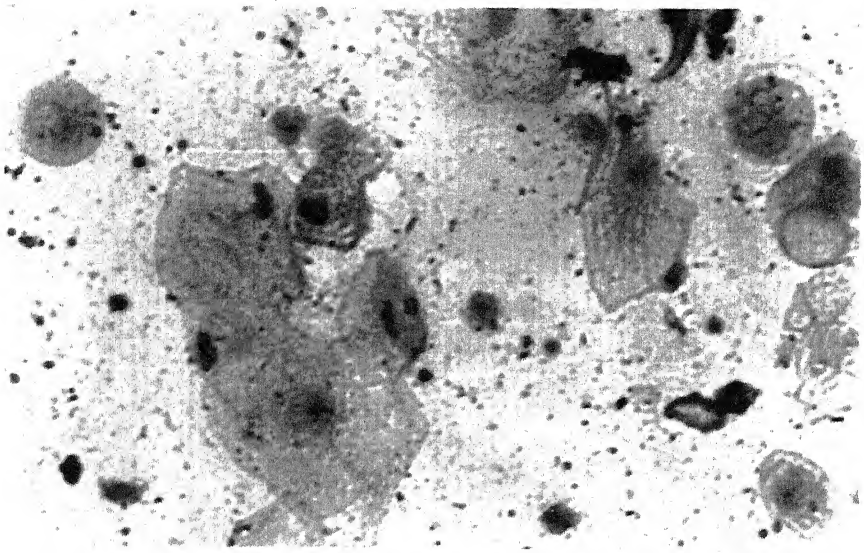


Figure-6.10: Photomicrographs showing smears of vaginal fluid containing partially cornified cells and few leukocytes in rat treated with proniosomal formulation containing ethinylestradiol after first day

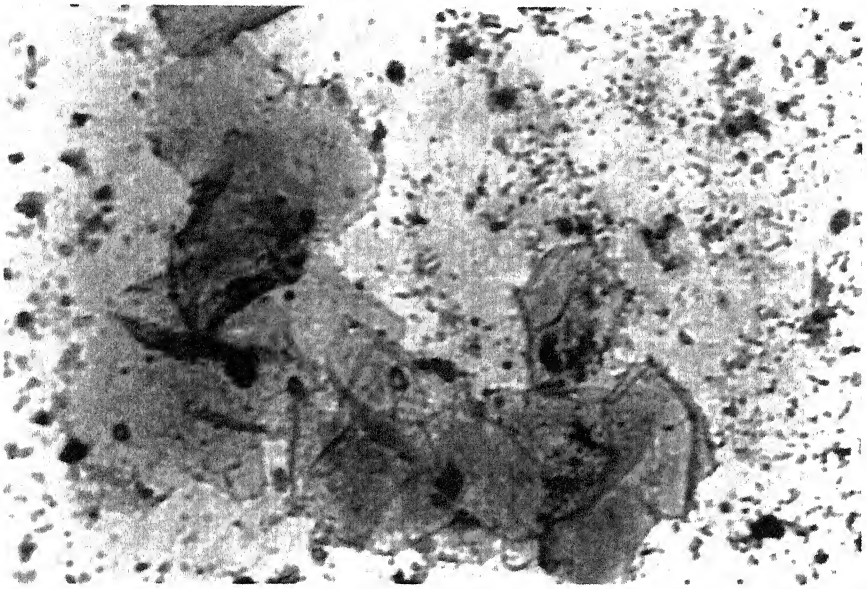


Figure-6.11: Photomicrographs showing smears of vaginal fluid containing cornified cells, no leukocytes in rat treated with proniosomal formulation containing ethinylestradiol after fourth day

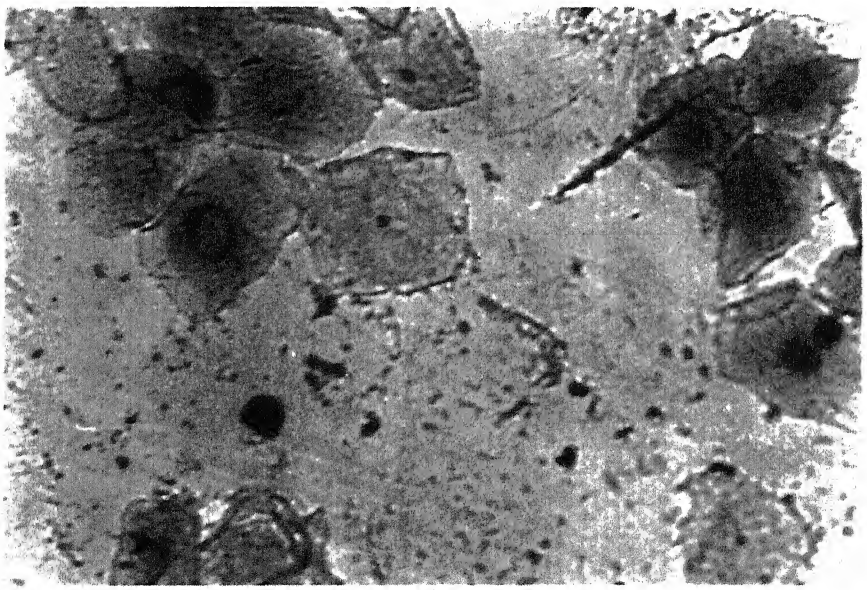


Figure-6.12: Photomicrographs showing smears of vaginal fluid containing only fully cornified cells, no leukocytes in rat treated with proniosomal formulation containing ethinylestradiol after seventh day

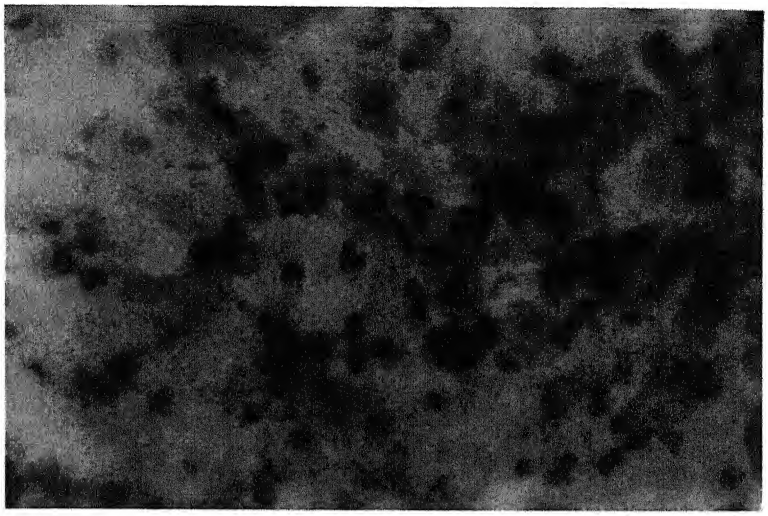


Figure-6.13: Photomicrographs showing smears of vaginal fluid containing leukocytes with some granulated cell and epithelium cell in rat treated with proniosomal formulation containing estradiol & levonorgestrel after first day

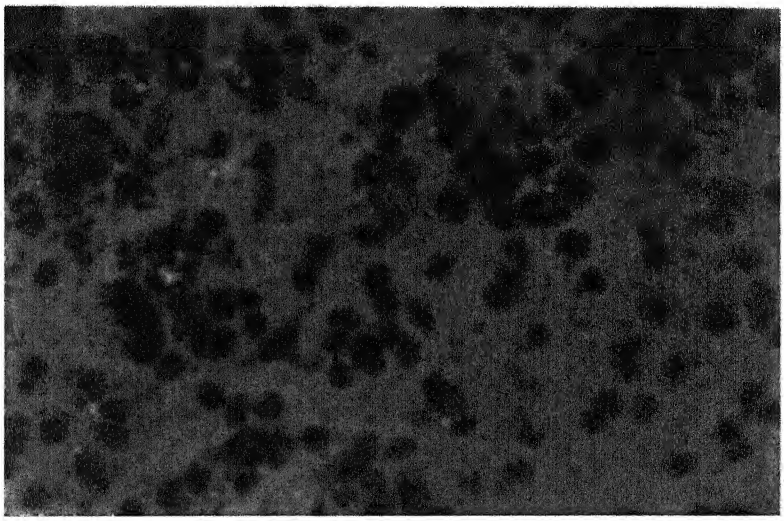


Figure-6.14: Photomicrographs showing smears of vaginal fluid containing leukocytes with some granulated cell and few epithelium cells in rat treated with proniosomal formulation containing estradiol& levonorgestrel after fourth day

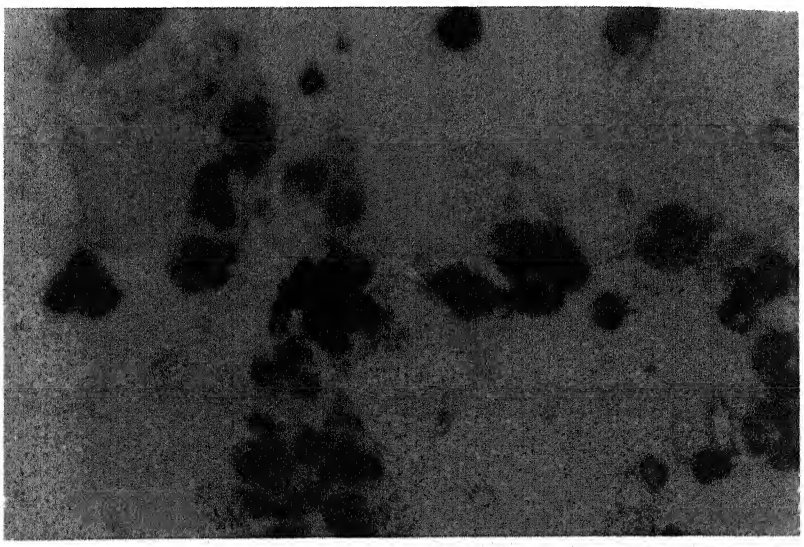


Figure-6.15: Photomicrographs showing smears of vaginal fluid containing leukocytes with some granulated cell and epithelium cell in rat treated with proniosomal formulation containing estradiol& levonorgestrel after seventh day

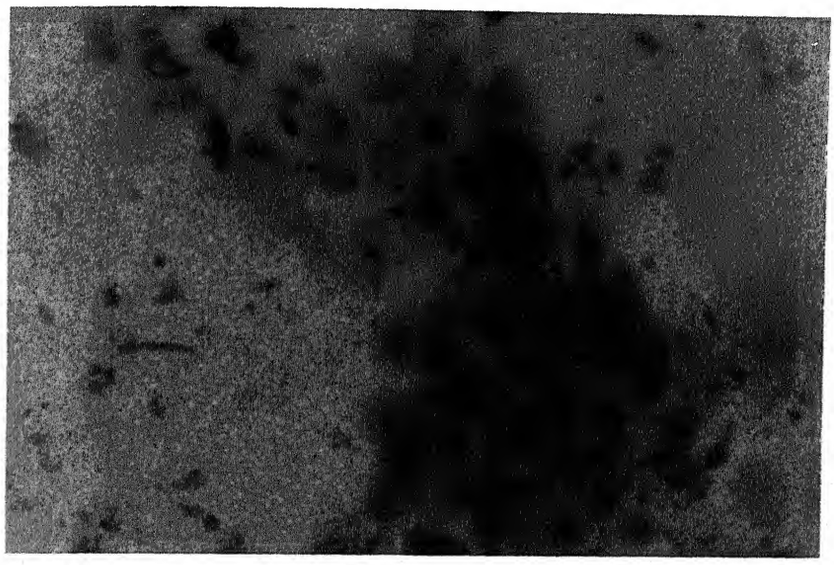


Figure-6.16: Photomicrographs showing smears of (viscous) vaginal fluid containing mixture of leukocytes and epithelium cell in rat treated with proniosomal formulation containing ethinylestradiol & levonorgestrel after first day

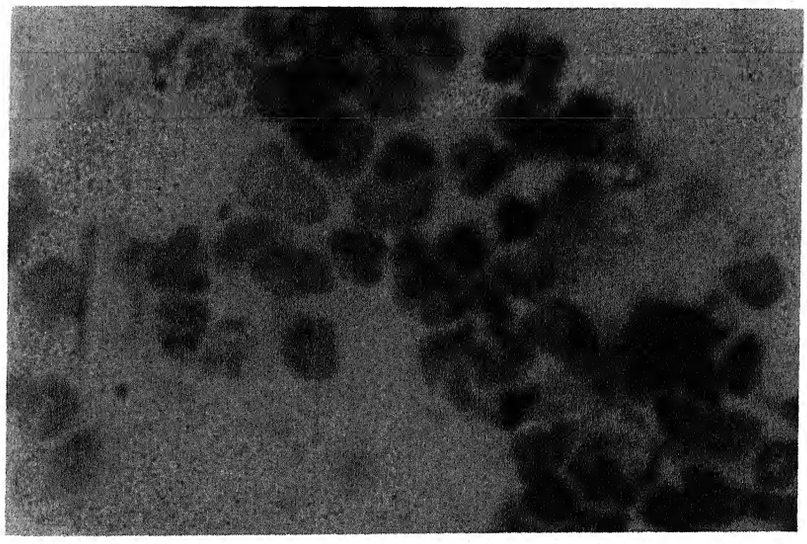


Figure-6.17: Photomicrographs showing smears of (viscous) vaginal fluid containing mixture of leukocytes and epithelium cell in rat treated with proniosomal formulation containing ethinylestradiol & levonorgestrel after fourth day

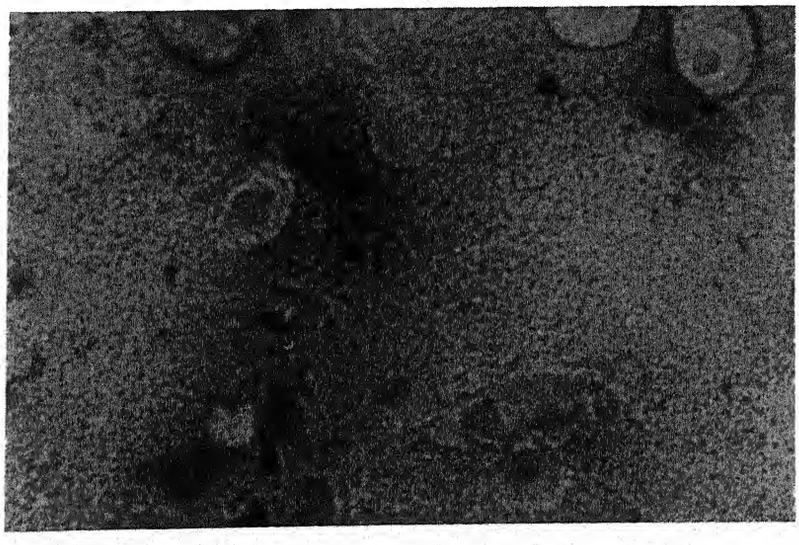


Figure-6.18: Photomicrographs showing smears of (viscous) vaginal fluid containing leukocytes and epithelium cell plus few cornified cell in rat treated with proniosomal formulation containing ethinylestradiol & levonorgestrel after seventh day

Smear Count was performed by the method of Bigger and Claringgold (1954). As discussed earlier, the value of normal animals (X=0) means in which some leukocytes granulated cells probably heading towards keratinization and only few thick cells are present. Mainly leukocytes were larger in number and epithelial cells were observed in the case of patches containing individual drug only while in the case of combined formulation treated animals, some progesterous smear, nucleated plus cornified cells (Y=1) were observed. But in the rats treated with formulation $T1_{(17\beta)}$ and $T1_{(EEE)}$ proniosomal gels, only epithelial cells were comparatively bigger than found in control animals. Finally many cornified cells with highly granulated with cytoplasm were visible, and defined picnosis was seen in the nuclei of some epithelial cells. Keratinized cells without any nucleus were also seen. No leukocytes could be seen in this stage. Non-keratinized cells that were clearly distinguished were also visible. This was the clear indication that the estradiol and ethinylestradiol produced full cornification.

Photomicrographs of histopathological study showed that the thickness of uterine mucosal thickness was increased and endometrial proliferation took place, which resulted in the increase of endometrium weight. The mucosal thickness in control animals treated with proniosomal gel $T1_{(Cont)}$, $T1_{(LN)}$, $T1_{(EEE)}$, and $T1_{(17\beta)}$, and combined formulation $T1_{(EEE + LN)}$ and $T1_{(17\beta + LN)}$ treated animals were 16.61 μm, 62.62 μm, 58.42 μm, 64.52 μm, and 70.12 μm and 76.32 μm respectively. Uterine mucosal thickness was 3.16 times and 4.59 times more in single drug formulation and in combined formulation respectively as compared to control animal. Histological picture of uterus shows that more, thick villi are present in uterine mucosa, and uterine glands are more in number (Photomicrographs 6.24, 6.30 and 6.27). Thus the proniosomal gel $T1_{(LN)}$, $T1_{(EEE)}$, containing levonorgestrel and ethinylestradiol shows more, but uniform effect on uterus than $T1_{(17\beta)}$ of estradiol and combined formulation $T1_{(EEE+LN)}$ and $T1_{(17\beta+LN)}$ of levonorgestrel + estradiol and levonorgestrel + ethinylestradiol.

CONTRACEPTIVE ACTIVITY:

The combined proniosomal gel formulation of selected drugs had several advantages over the existing hormonal contraceptive preparations. They proved to be a novel and controlled release, contraceptive preparation. A number of implantable drug delivery systems have been reported for a variety of contraceptive drugs. The present investigation examined proniosomal-gel-based-long-acting–transdermal-patch system for

(i) levonorgestrel (ii) ethinylestradiol and levonorgestrel (iii) ethinylestradiol and levonorgestrel. It is non-toxic and biodegradable in nature and may be extensively used to achieve controlled release of this proniosomal drug delivery of $T1_{(LN)}$, $T1_{(EEE+LN)}$ and $T1_{(17\beta+LN)}$ formulation to achieve desired contraceptive effect and supplement for estrogenic deficiency.

Assay of contraceptive formulation for showing the effect on corpora lutea:

Endometrium assay: The morphological changes produced by the direct action of combined formulation on the uterine endometrium of the rats were recorded. The uterine thickness, lumen, myometrium and effect on villi formation in each group of rats were determined using calibrated ocular micrometer (Elico Instrument Hyderabad). Photomicrographs of selected sections were taken using microscope fitted with a photographic attachment (Leitz Germany). Both, the area of the mucosal and glandular part of the endometrium were also determined by tracing the thickness in photomicrographs of selected sections. In all proniosomal formulation containing estradiol was showed very week effect on endometrium but uniform in thickness. The levonorgestrel effect, however, varied greatly and with in the whole sample. In three samples the effect was very week with only some subnuclear vacuoles seen in the glandular epithelium. In these samples the response of the endometrium to the levonorgestrel was good although focal, with some of the glands showing secretion with vacuoles both above and below the nuclei or secreted material in the lumina but with some glands showing no sign of secretional activity. The Observations are recorded in table 6.1-6.2 and endometrium section of uterus shown in photomicrographs 6.19-6.36.

RESULT AND DISCUSSION:

Contraceptive activity: From the pictures (Photomicrographs 6.49-6.54) and table (6.1-6.2). It is clear that the application of combined proniosomal gel bearing levonorgestrel, ethinylestradiol + levonorgestrel and estradiol + levonorgestrel resulted in the inhibition of formation of corpora lutea, which was consequences of inhibition of LH (Leuteinizing hormone) as described by Hebborn. Moreover the ovulation points were not observed in animals bearing transdermal patches containing both drugs indicating that the formulation had shown antiovulatory activity.

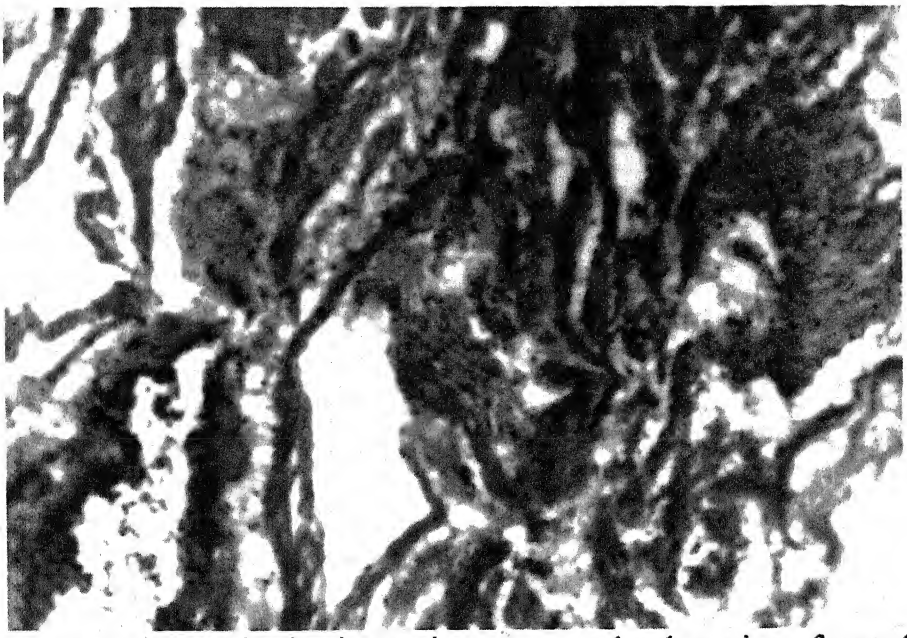


Figure-6.19: Photomicrographs showing uterine mucosa and endometrium of control rat after first day

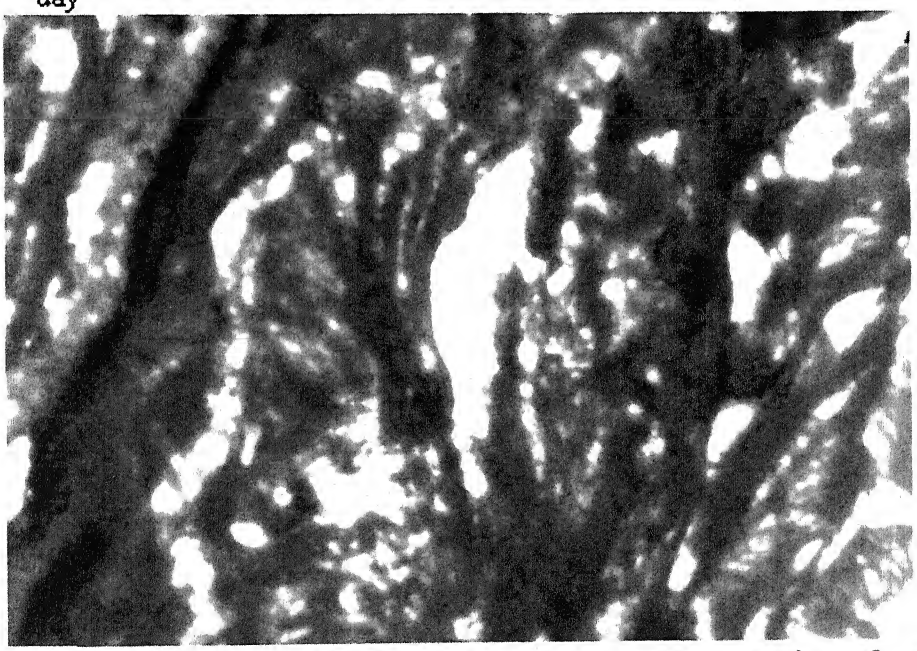


Figure-6.20: Photomicrographs showing uterine mucosa and endometrium of control rat after fourth day

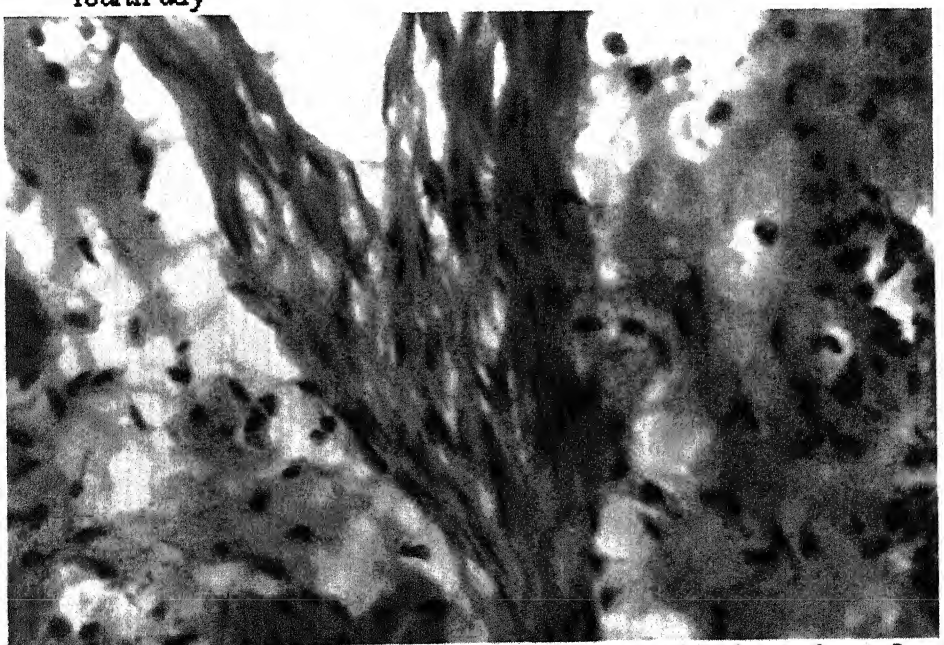


Figure-6.21: Photomicrographs showing uterine mucosa and endometrium of control rat after seventh day

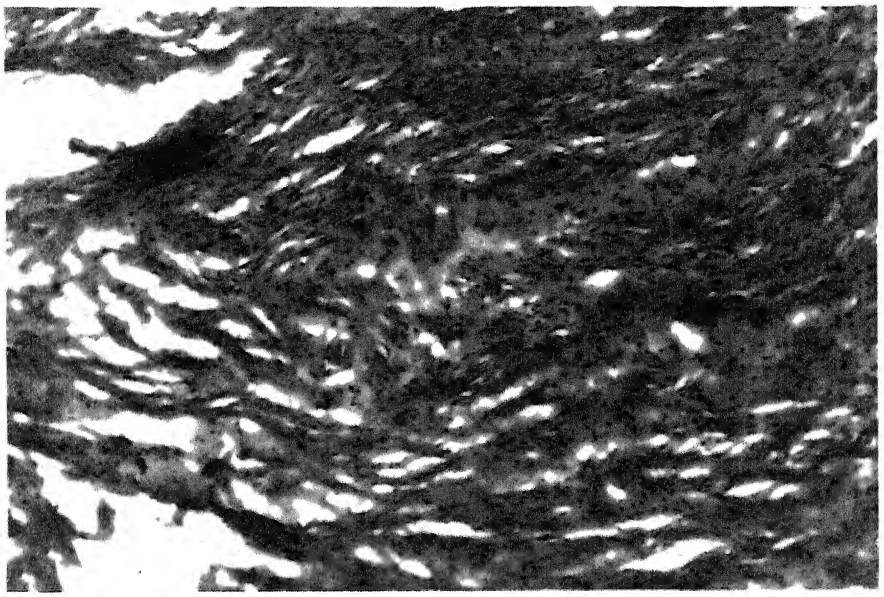


Figure-6.22: Photomicrographs showing uterine mucosa and endometrium of rat treated with proniosomal formulation containing levonorgestrel after first day

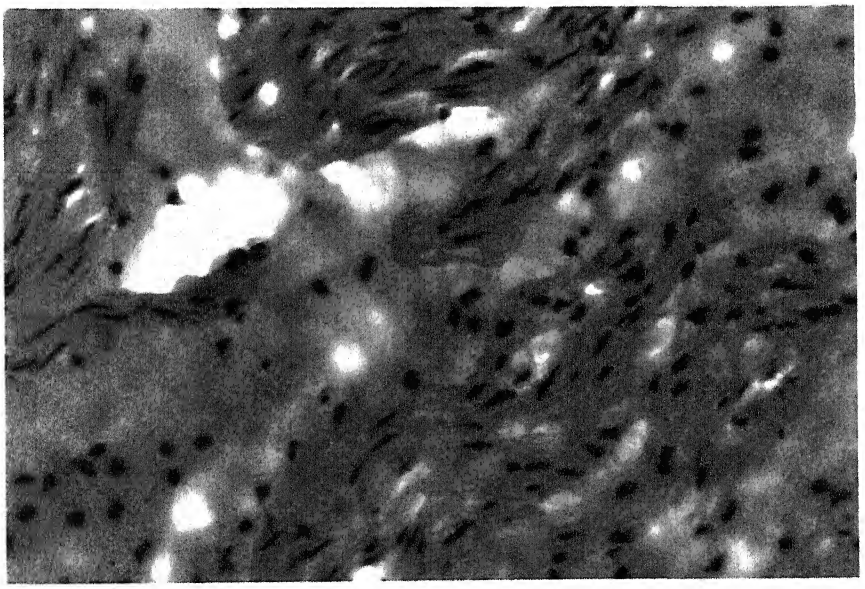


Figure-6.23: Photomicrographs showing uterine mucosa and endometrium of rat treated with proniosomal formulation containing levonorgestrel after fourth day

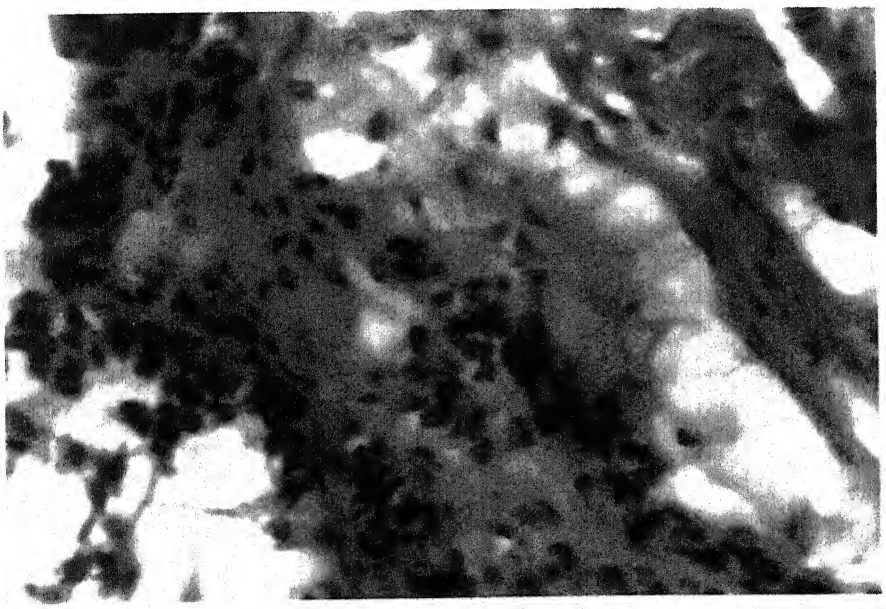


Figure-6.24: Photomicrographs showing uterine mucosa and endometrium of rat treated with proniosomal formulation containing levonorgestrel after seventh day

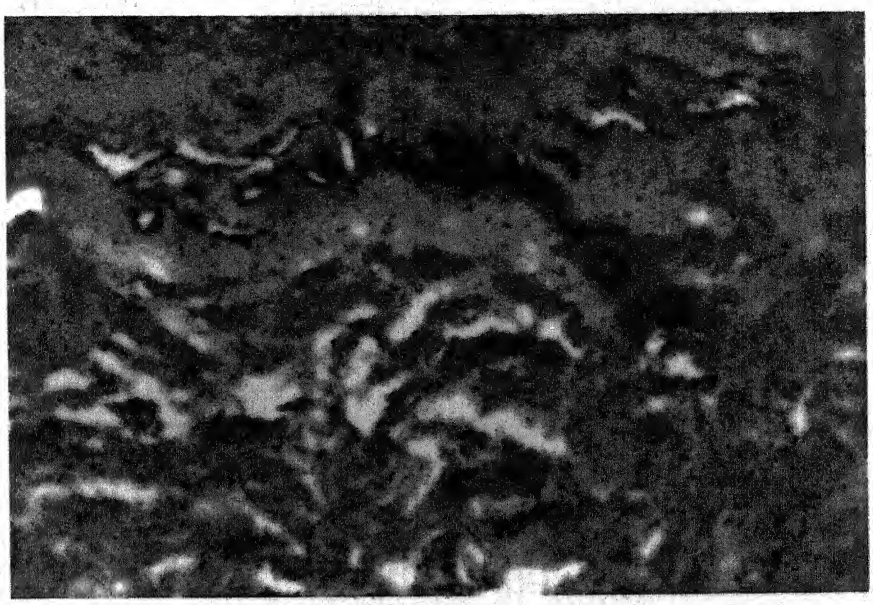


Figure-6.25: Photomicrographs showing uterine mucosa and endometrium of rat treated with proniosomal formulation containing estradiol after first day

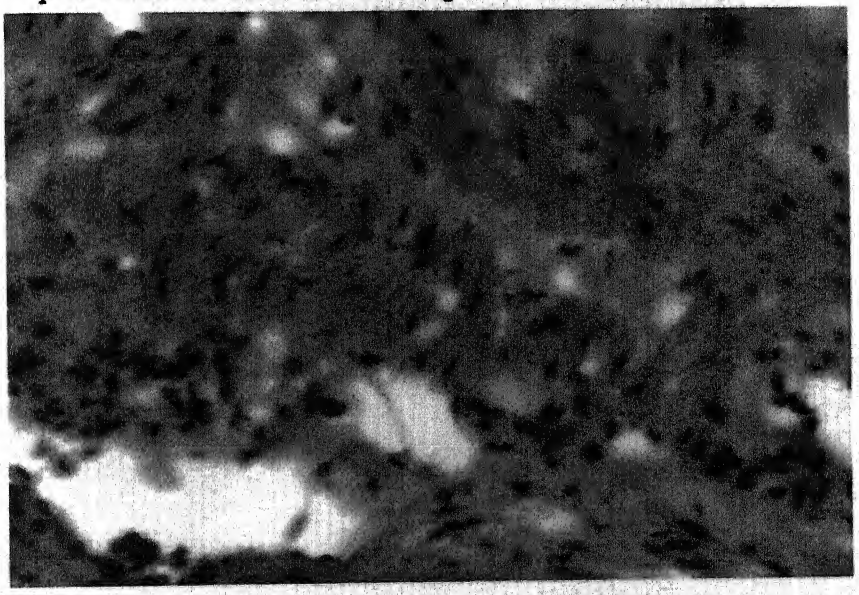


Figure-6.26: Photomicrographs showing uterine mucosa and endometrium of rat treated with proniosomal formulation containing estradiol after fourth day

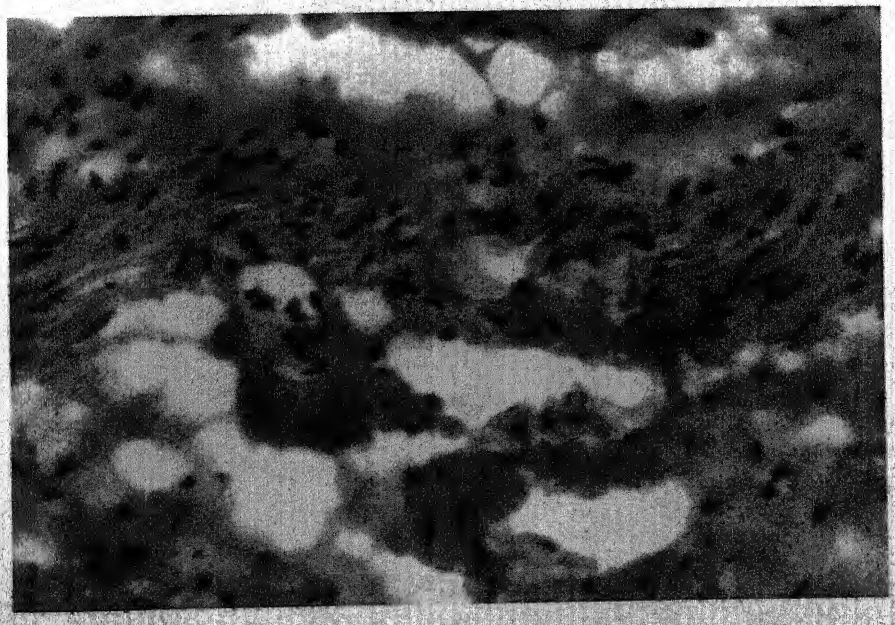


Figure-6.27: Photomicrographs showing uterine mucosa and endometrium of rat treated with proniosomal formulation containing estradiol after seventh day

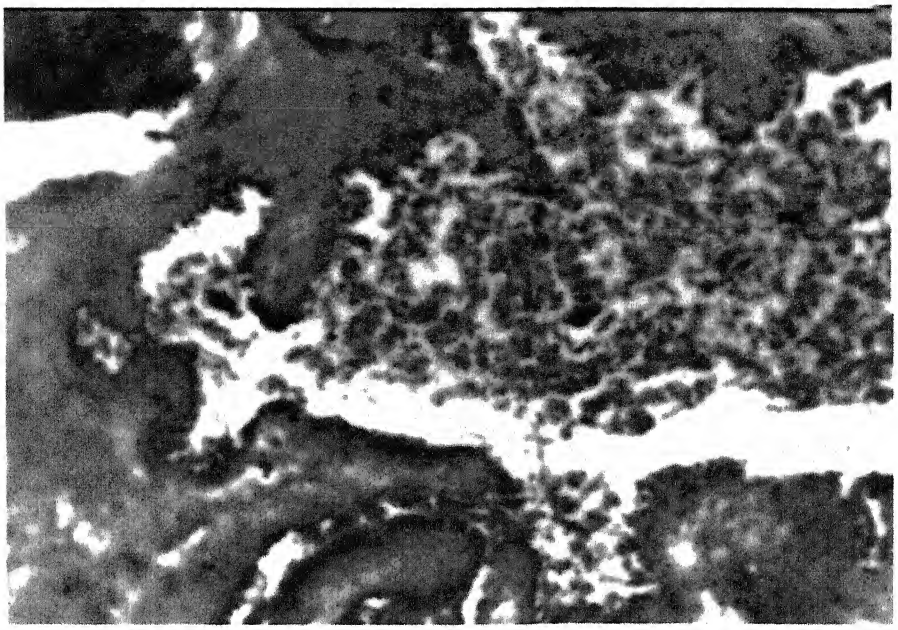


Figure-6.28: Photomicrographs showing uterine mucosa and endometrium of rat treated with proniosomal formulation containing ethinylestradiol after first day

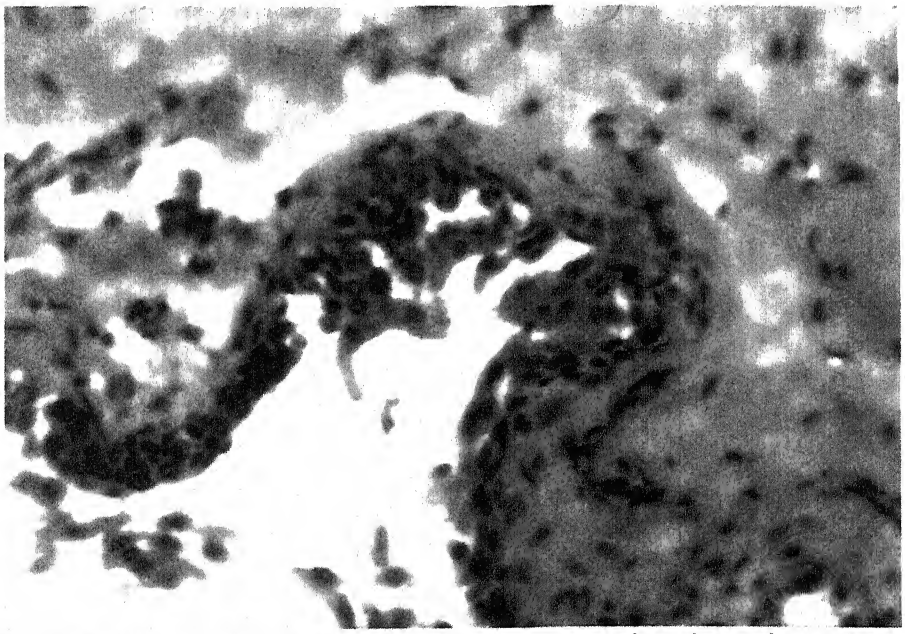


Figure-6.29: Photomicrographs showing uterine mucosa and endometrium of rat treated with proniosomal formulation containing ethinylestradiol after fourth day

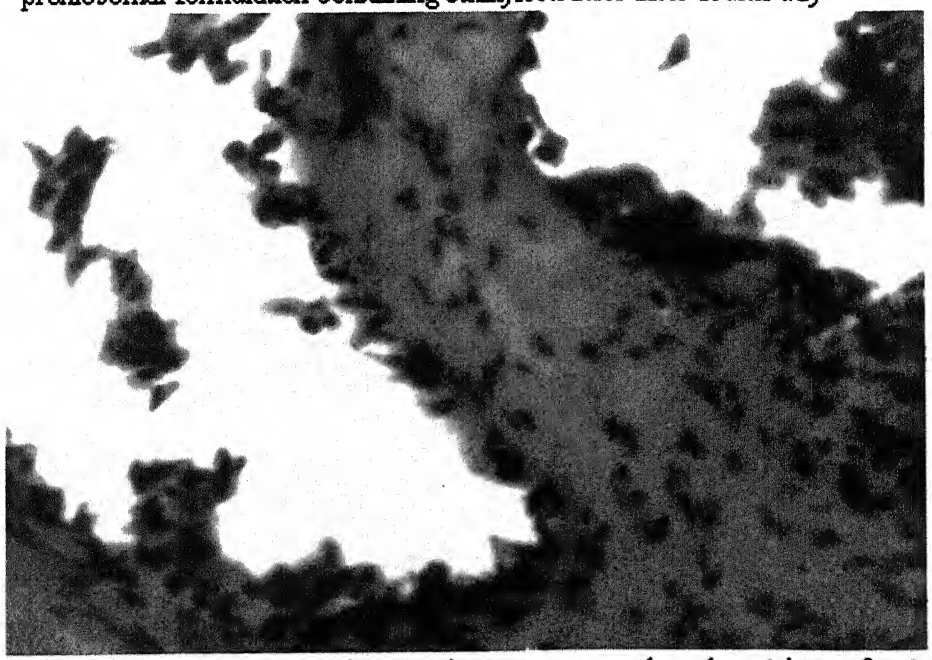


Figure-6.30: Photomicrographs showing uterine mucosa and endometrium of rat treated with proniosomal formulation containing ethinylestradiol after seventh day

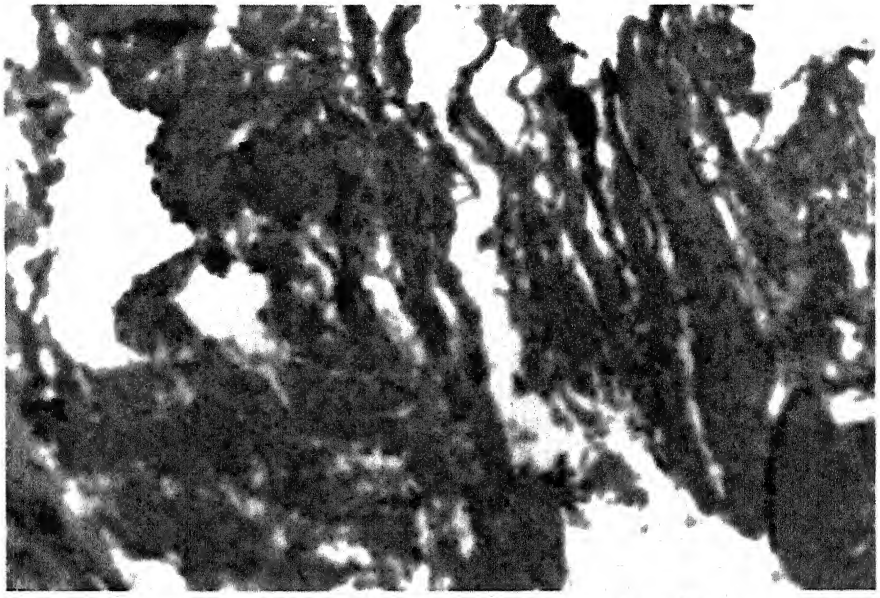


Figure-6.31: Photomicrographs showing uterine mucosa and endometrium of rat treated with proniosomal formulation containing estradiol& levonorgestrel after first day

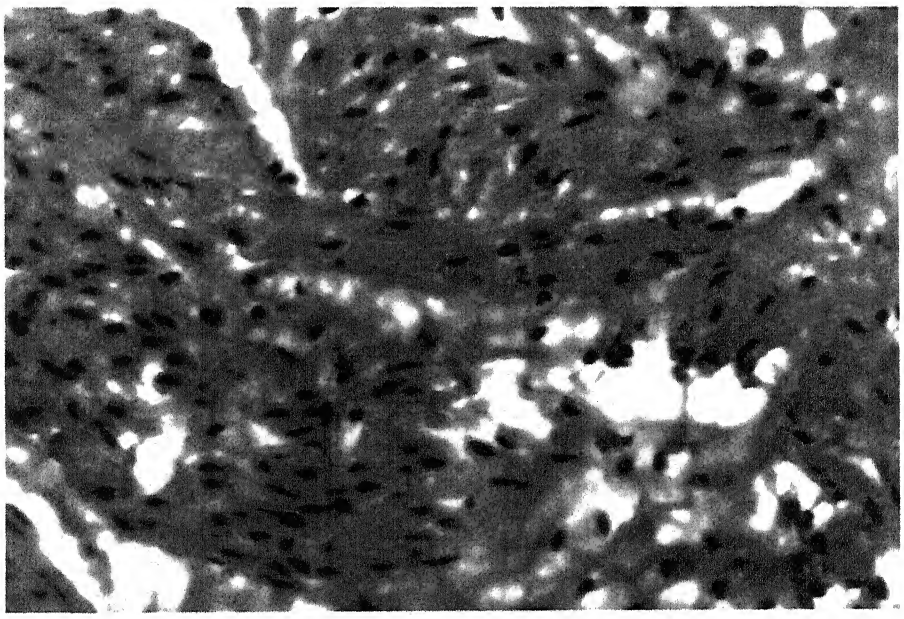


Figure-6.32: Photomicrographs showing uterine mucosa and endometrium of rat treated with proniosomal formulation containing estradiol& levonorgestrel after fourth day

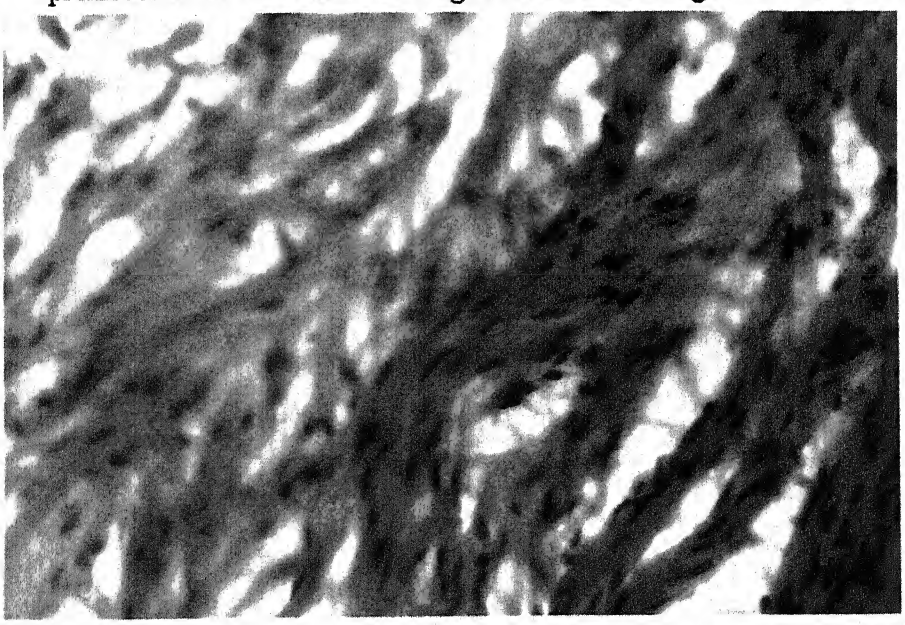


Figure-6.33: Photomicrographs showing uterine mucosa and endometrium of rat treated with proniosomal formulation containing estradiol& levonorgestrel after seventh day

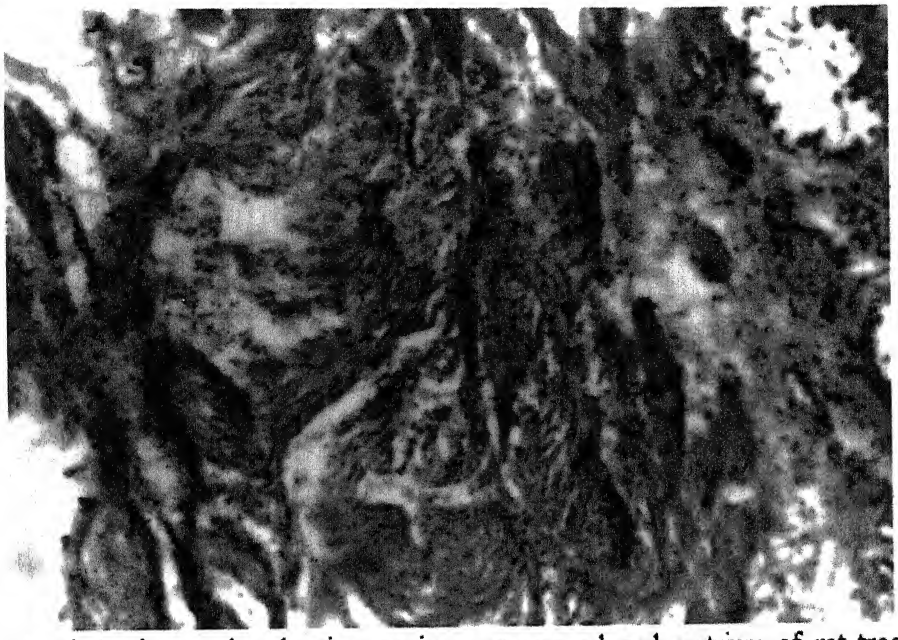


Figure-6.34: Photomicrographs showing uterine mucosa and endometrium of rat treated with proniosomal formulation containing ethinylestradiol& levonorgestrel after first day

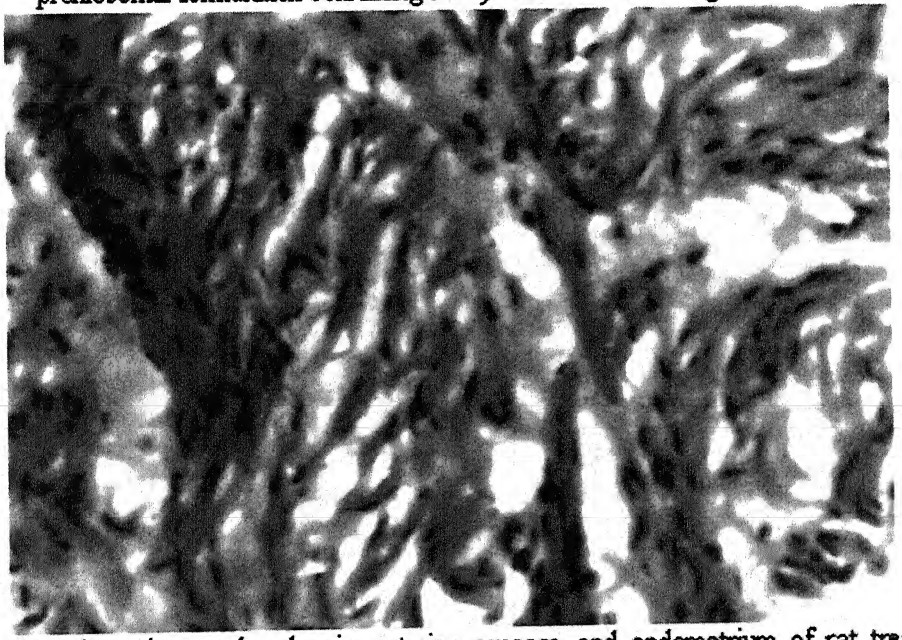


Figure-6.35: Photomicrographs showing uterine mucosa and endometrium of rat treated with proniosomal formulation containing ethinylestradiol& levonorgestrel after fourth day

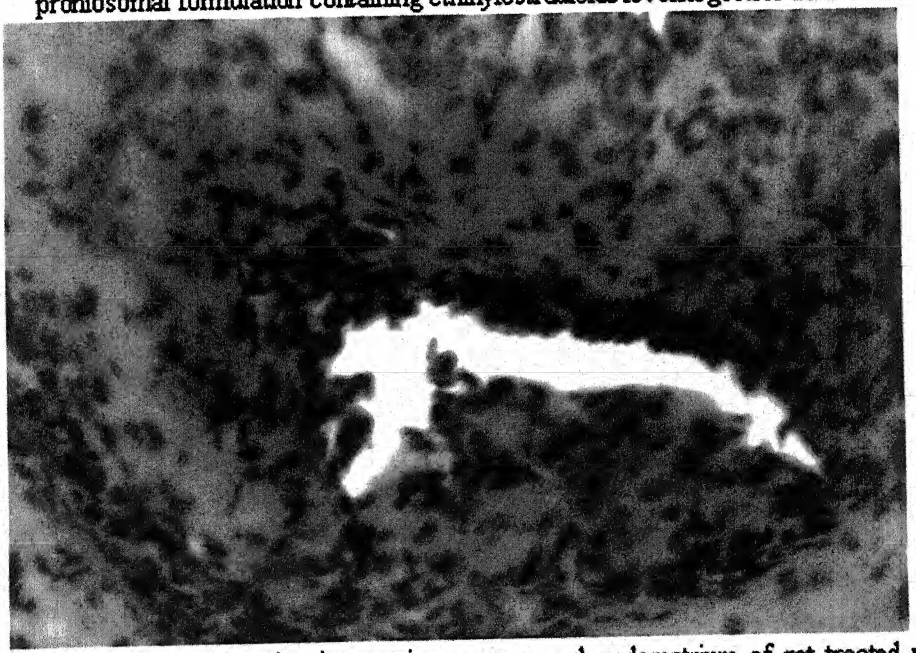


Figure-6.36: Photomicrographs showing uterine mucosa and endometrium of rat treated with proniosomal formulation containing ethinylestradiol& levonorgestrel after seventh day

The interference in the formation of corpora lutea was greater in both combined formulation $T1_{(EEE+LN)}$ and $T1_{(17\beta+LN)}$ than $T1_{(LN)}$, $T1_{(EEE)}$, $T1_{(17\beta)}$ formulation. The percent inhibition with combined proniosomal gel of estradiol + levonorgestrel and ethinylestradiol + levonorgestrel treated rats was more than those treated with proniosomal gel containing single drug. These observations confirmed the controlled and prolonged delivery of combined formulations as contraceptive patch.

As shown in photomicrograph, the combined formulation of estradiol + levonorgestrel and ethinylestradiol + levonorgestrel provides contraception by producing or altering morphology of ovaries, uterine mucosa and endometrium by inhibiting the secretion of leuteinizing hormone (LH) which required for ovulation and FSH which is required for transformation of Graffian follicle into a functioning of corpus luteum.

These studies have clearly shown that the developed transdermal patches bearing proniosomal gels of levonorgestrel, estradiol + levonorgestrel and ethinylestradiol + levonorgestrel continuously delivered these drugs within therapeutic level upto 07 days for contraception purpose. Thus proniosomal gel bearing levonorgestrel, estradiol + levonorgestrel and ethinylestradiol + levonorgestrel have proved to be satisfactory drug delivery system for its use as contraceptives.

Table 6.1: Effect of formulation on uterine weight, vaginal opening and vaginal cornification.

S. No.	Animals Group	Uterine weight	Vaginal opening	Vaginal Cornification	Mucosal Thickness
1.	$T1_{(Control)}$	99.5±6.66	0.00%	0.00%	16.61
2.	$T1_{(Levonorgestrel)}$	111.4±4.82	74%	88%	62.62
3.	$T1_{(17\beta\text{-Estradiol})}$	128.5±3.69	100%	100%	58.42
4.	$T1_{(Ethinylestradiol)}$	209.6±4.42	98%	90%	64.52
5.	$T1_{(LN+EEE)}$	174.6±7.35	66%	76%	70.12
6.	$T1_{(LN+17\beta E)}$	189.8±5.88	61%	82%	76.32

Table 6.2: Inhibition of Leuteinization in rats' ovary by different formulation

S. No.	Animals Group	Dose	No. of rats with OP	No. of Rats with CL	Mean No. of CL	Percent inhibition
1.	$T1_{(Control)}$	--	6	6	16	---
2.	$T1_{(Levonorgestrel)}$	1 mg	6	5	6	62.5%
3.	$T1_{(17\beta\text{-Estradiol})}$	1 mg	6	4	9	43.75%
4.	$T1_{(Ethinylestradiol)}$	1 mg	6	4	8	50.00%
5.	$T1_{(LN+EEE)}$	1+1 mg	3	1	2	87.5%
6.	$T1_{(LN+17\beta E)}$	1+1mg	2	1	1	93.75%

GENERAL DISCUSSION:

Effect on ovary: The antiovulatory effect of estradiol and ethinylestradiol on the ovary and uterus can be compared with normal rat's ovary and uterus. In the section of the ovary all conditions of developing ovum were present in the slide of normal animal. The stroma, developing oocytes, larger blood vessels and compressed cell of stroma were seen, almost in every stage, the Graffian follicles were present and ovum surrounded by granular cells. But one day after application of transdermal patch there was no change except thicka externa was not uniform and was wavy at some places. Secondly, there was no any new formation of ova and all these layers were also not seen properly. Thirdly, germinal epithelium was not active, reduced the primary follicle and space were developed around the developing oocytes which may be due to lysis of cells. These all effects on the ovary may be due to the effect of estradiol and ethinylestradiol on the FSH, which brought changes into the ovulation (Photomicrographs 6.43-6.48).

Effect on Uterus: The histology of normal uterus showed a lumen divided into many partitions due to the formation of fold in the myometrium. It was covered by two-three layers thick epithelial endometrial cells are oval with small nucleus at few places, cells were surrogated in few uterine villi as the cells were dividing uterus in preparative or proliferative phase. The effect of estradiol and ethinylestradiol on these structure was more in comparison to levonorgestrel alone or both combined formulations i.e. estradiol + levonorgestrel and ethinylestradiol + levonorgestrel. This may be due to the estradiol and ethinylestradiol, which had increased the protein synthesis of endometrium wall. The clear effect of estradiol and ethinylestradiol was indicated by the increase in thickness

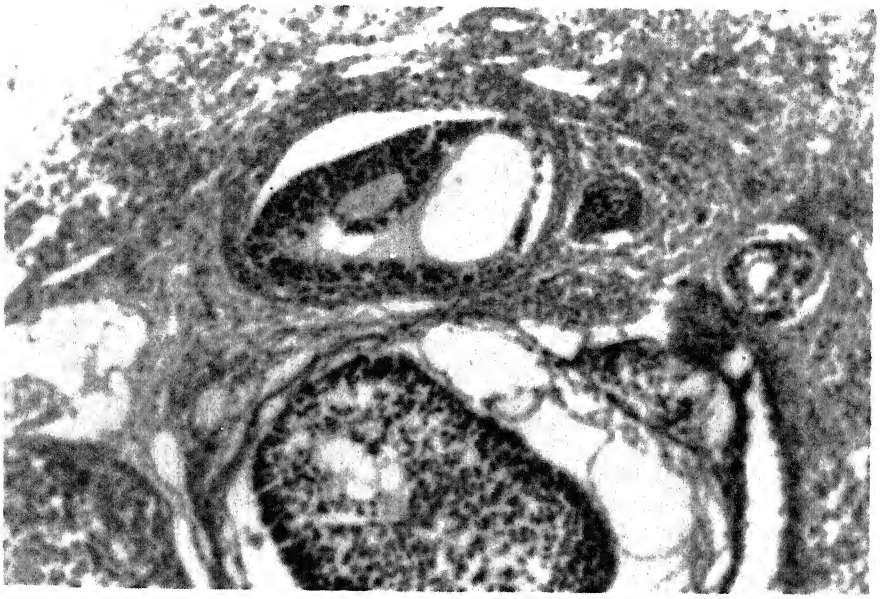


Figure-6.37: Photomicrographs showing corpora lutea with ovulation point formed in ovary of control rat after first day

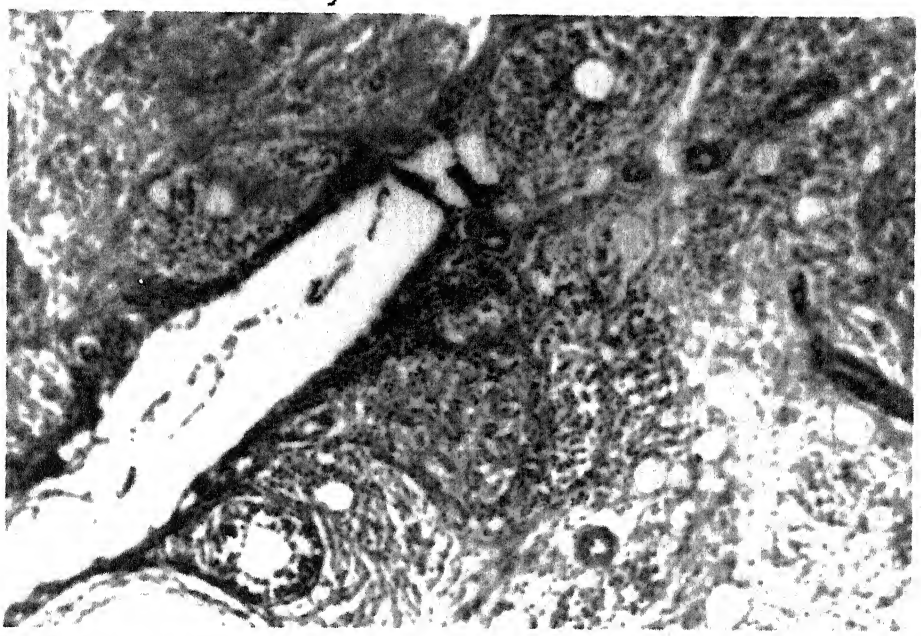


Figure-6.38: Photomicrographs showing corpora lutea with ovulation point formed in ovary of control rat after fourth day

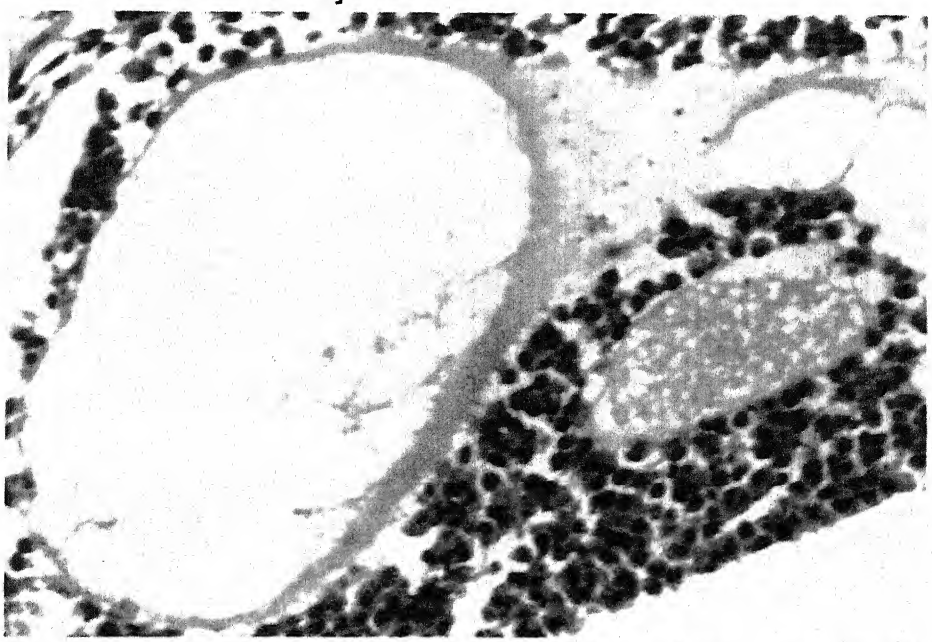


Figure-6.39: Photomicrographs showing corpora lutea with ovulation point formed in ovary of control rat after seventh day

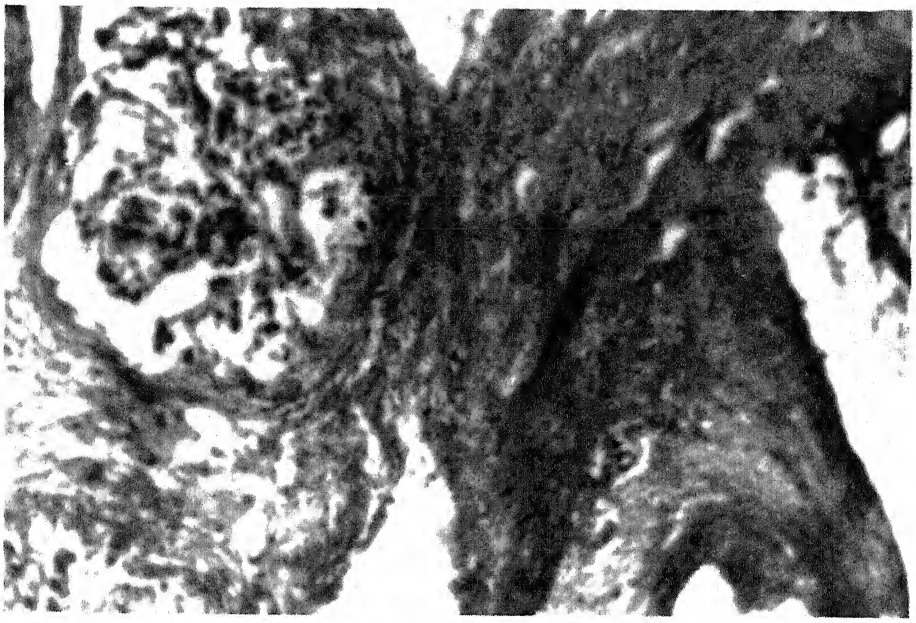


Figure-6.40: Photomicrographs showing corpora lutea with ovulation point formed in ovary of rat treated with proniosomal formulation containing levonorgestrel after first day

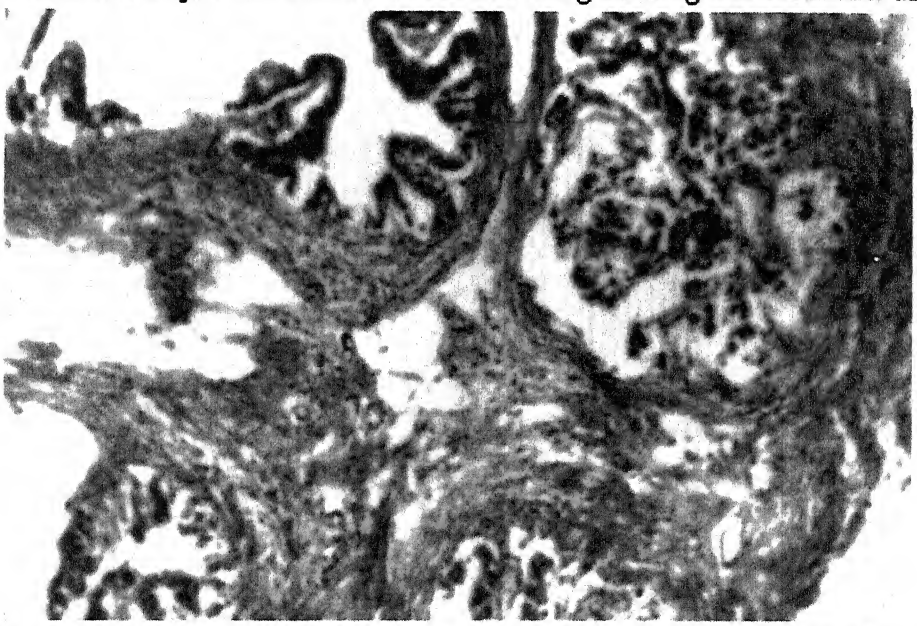


Figure-6.41: Photomicrographs showing corpora lutea with ovulation point formed in ovary of rat treated with proniosomal formulation containing levonorgestrel after fourth day

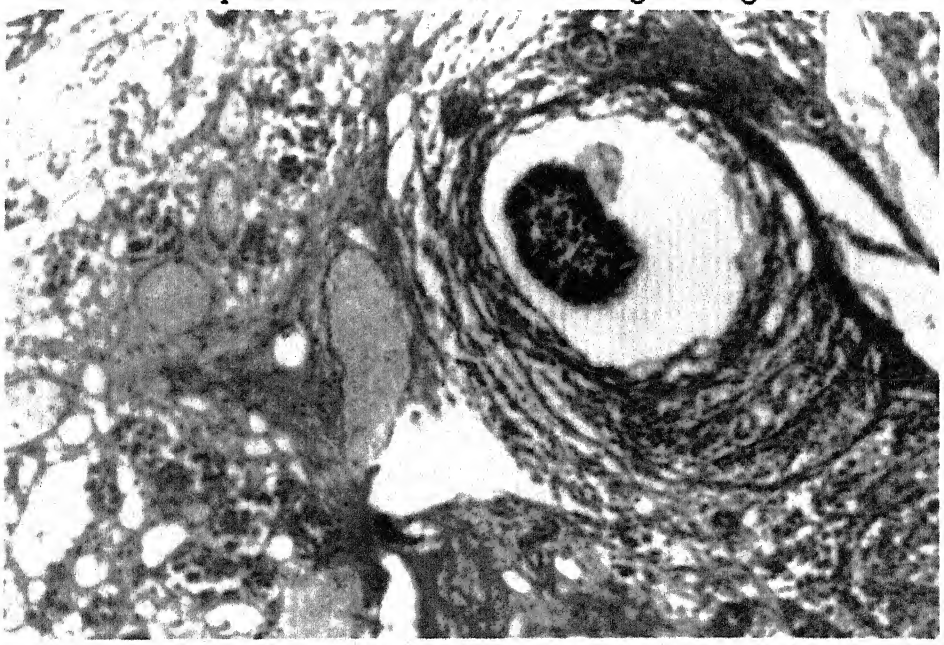


Figure-6.42: Photomicrographs showing corpora lutea with ovulation point formed in ovary of rat treated with proniosomal formulation containing levonorgestrel after seventh day

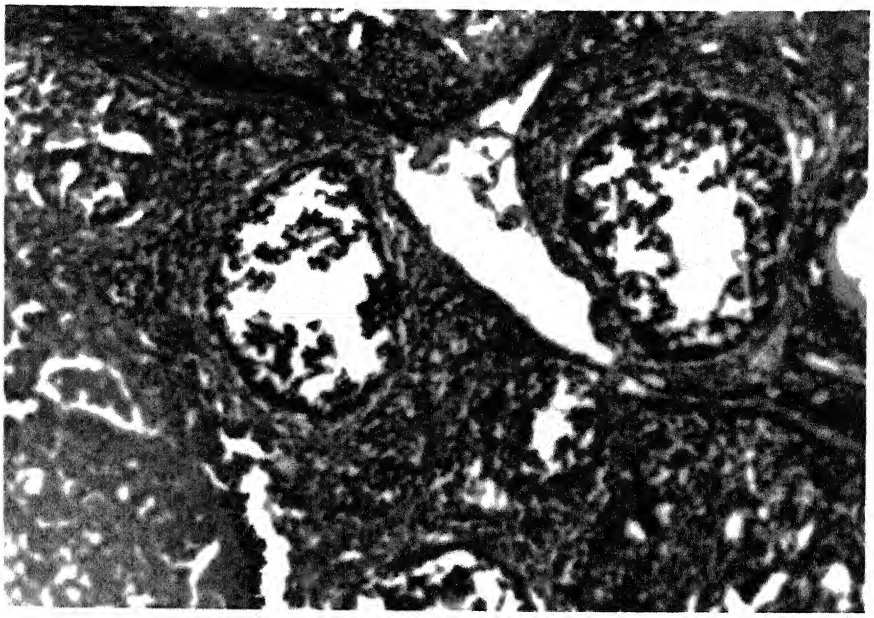


Figure-6.43: Photomicrographs showing corpora lutea with ovulation point formed in ovary of rat treated with proniosomal formulation containing estradiol after first day

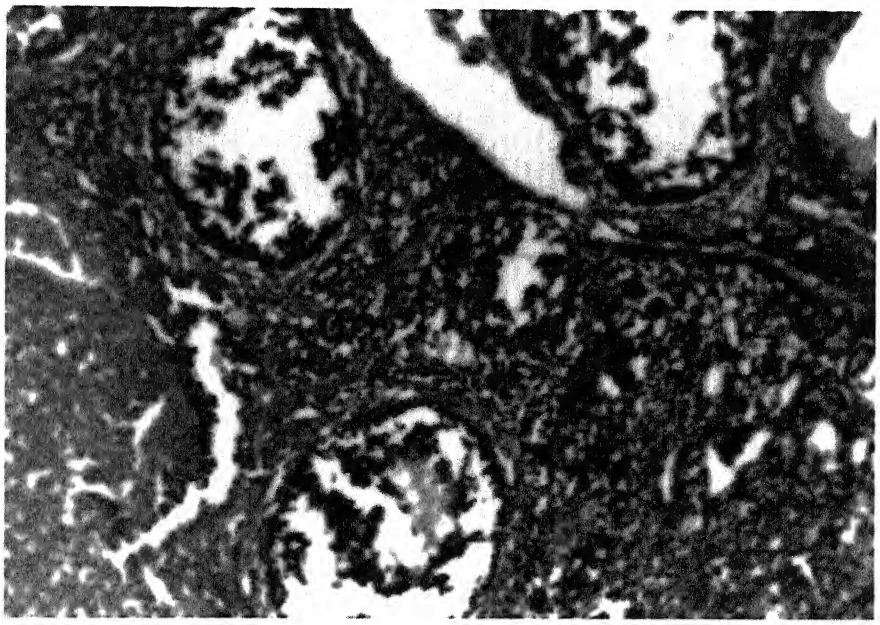


Figure-6.44: Photomicrographs showing corpora lutea with ovulation point formed in ovary of rat treated with proniosomal formulation containing estradiol after fourth day

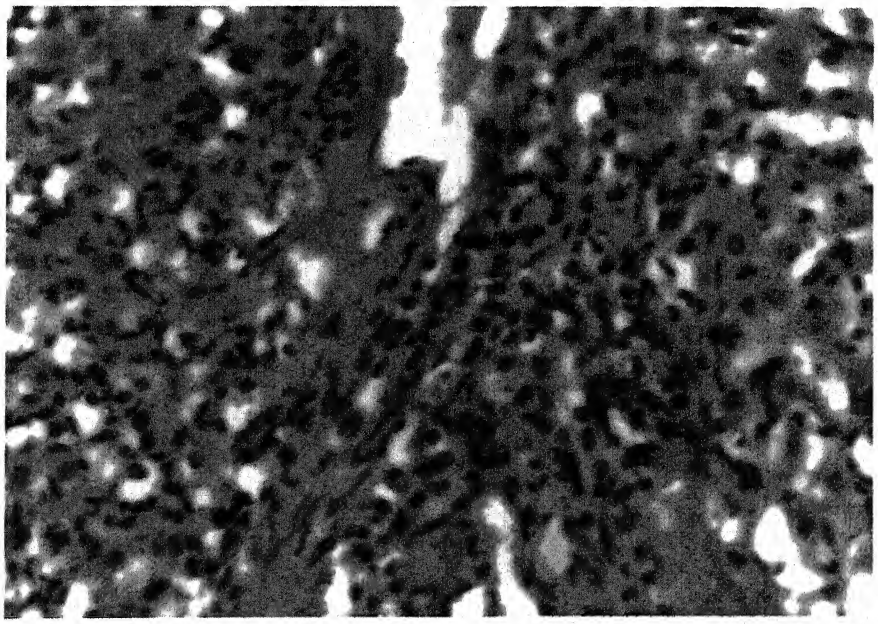


Figure-6.45: Photomicrographs showing corpora lutea with ovulation point formed in ovary of rat treated with proniosomal formulation containing estradiol after seventh day

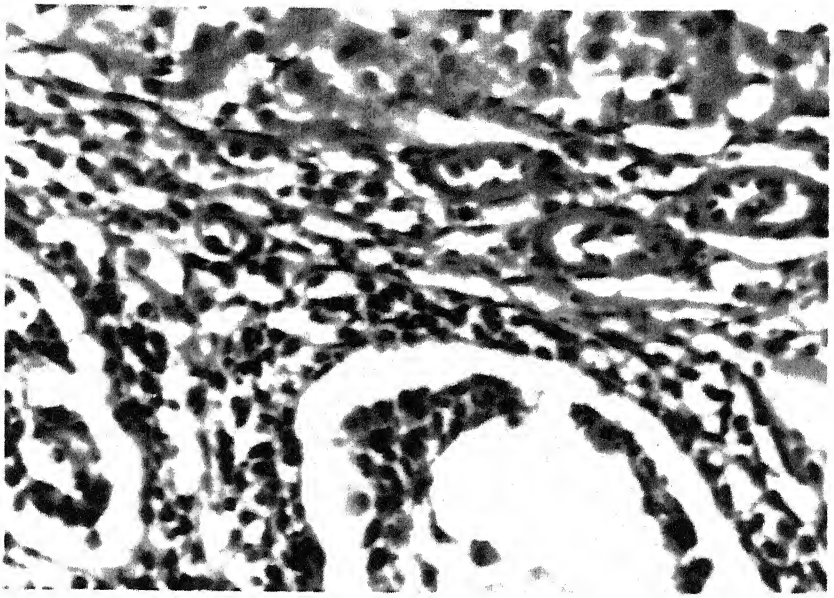


Figure-6.46: Photomicrographs showing corpora lutea with ovulation point formed in ovary of rat treated with proniosomal formulation containing ethinylestradiol after first day

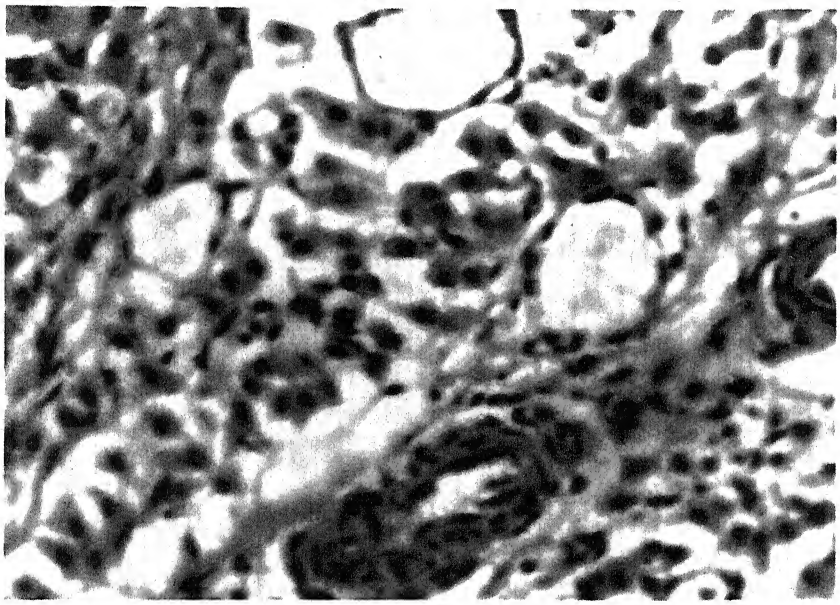


Figure-6.47: Photomicrographs showing corpora lutea with ovulation point formed in ovary of rat treated with proniosomal formulation containing ethinylestradiol after fourth day

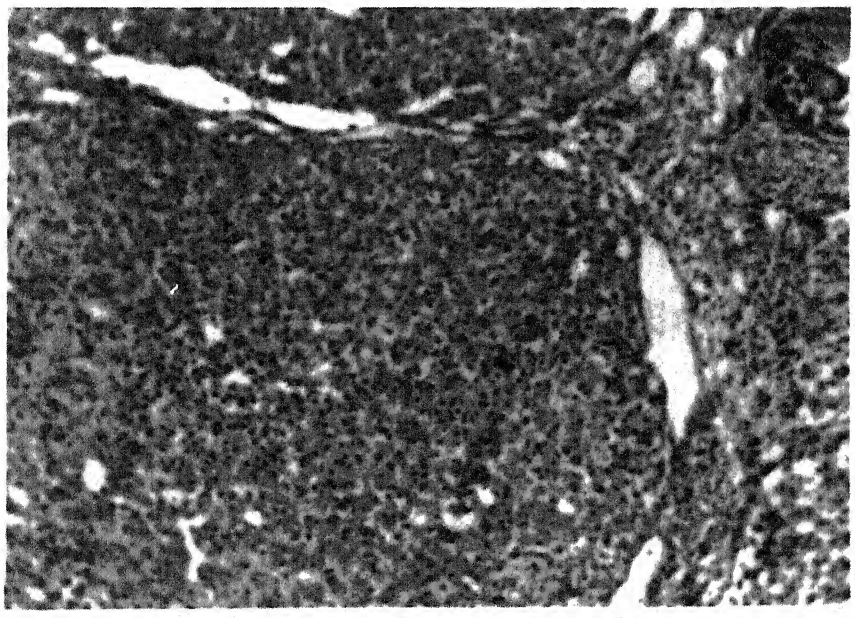


Figure-6.48: Photomicrographs showing corpora lutea with ovulation point formed in ovary of rat treated with proniosomal formulation containing ethinylestradiol after seventh day

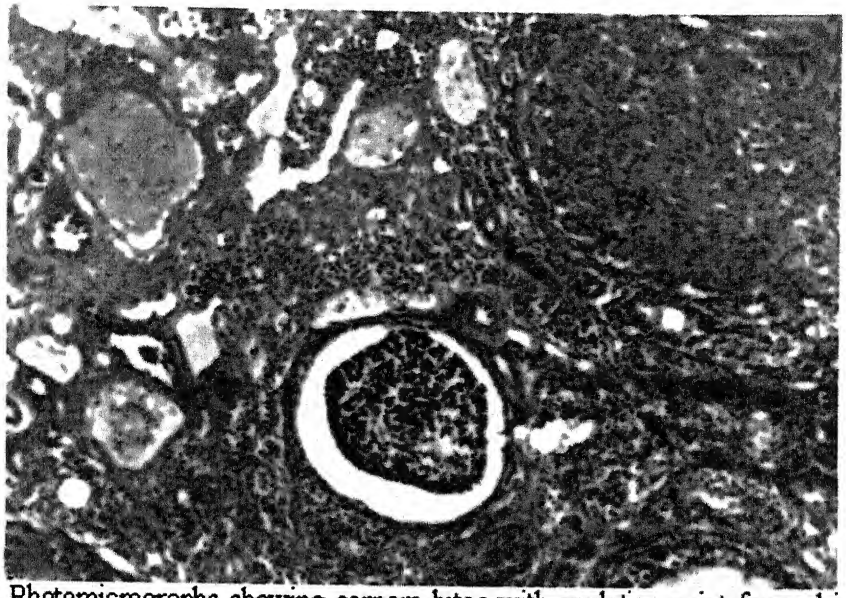


Figure-6.49: Photomicrographs showing corpora lutea with ovulation point formed in ovary of rat treated with proniosomal formulation containing estradiol& levonorgestrel after first day

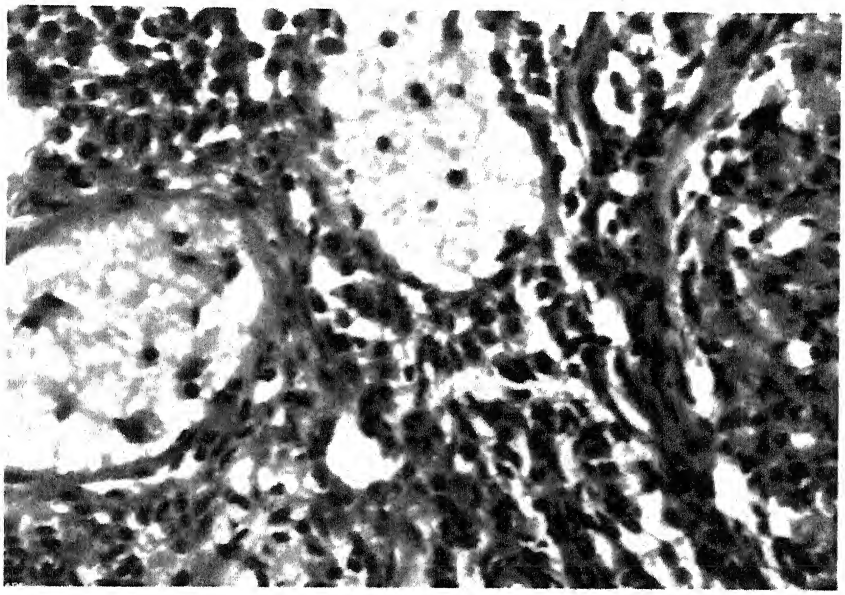


Figure-6.50: Photomicrographs showing corpora lutea with ovulation point formed in ovary of rat treated with proniosomal formulation containing estradiol& levonorgestrel after fourth day

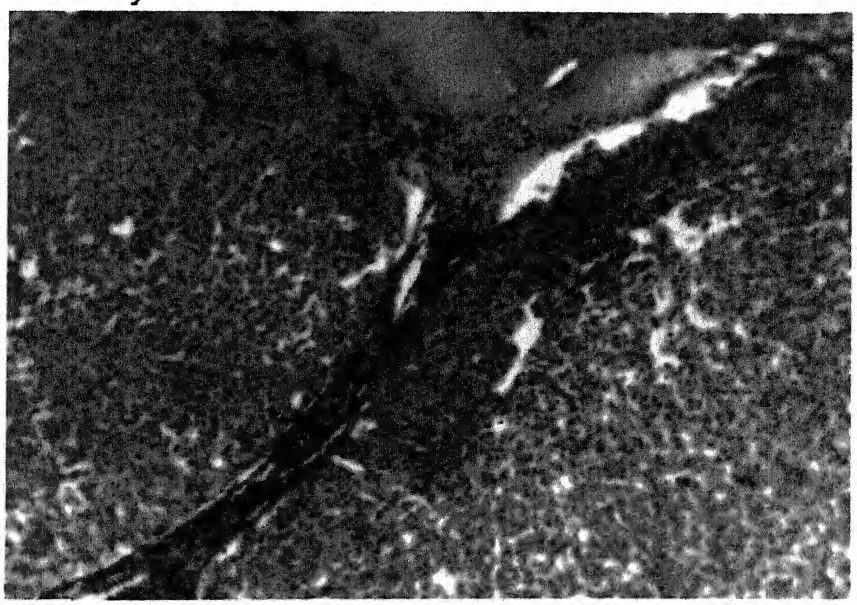


Figure-6.51: Photomicrographs showing corpora lutea with ovulation point formed in ovary of rat treated with proniosomal formulation containing estradiol& levonorgestrel after seventh day

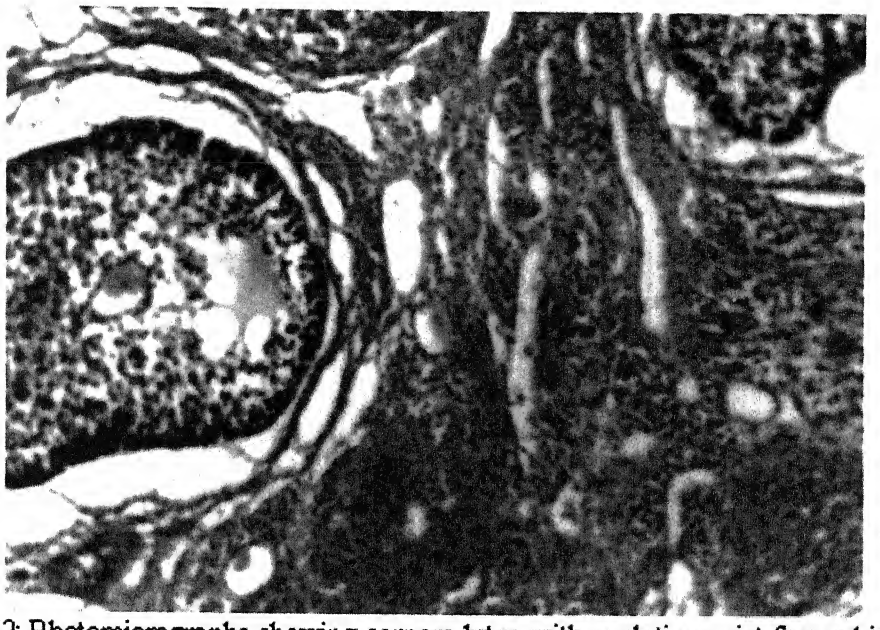


Figure-6.52: Photomicrographs showing corpora lutea with ovulation point formed in ovary of rat treated with proniosomal formulation containing ethinylestradiol& levonorgestrel after first day

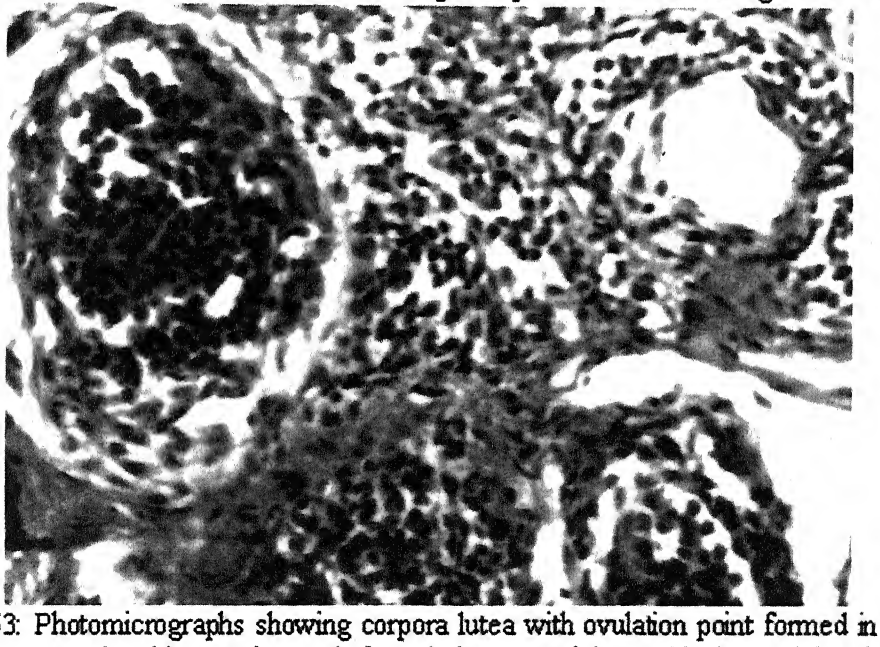


Figure-6.53: Photomicrographs showing corpora lutea with ovulation point formed in ovary of rat treated with proniosomal formulation containing ethinylestradiol& levonorgestrel after fourth day

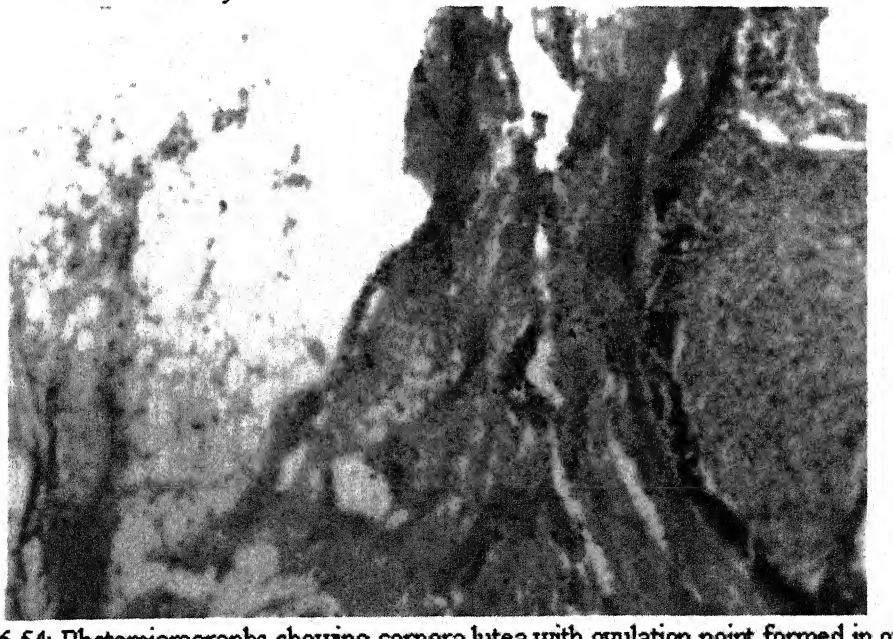


Figure-6.54: Photomicrographs showing corpora lutea with ovulation point formed in ovary of rat treated with proniosomal formulation containing ethinylestradiol& levonorgestrel after seventh day

and number of more villi present and higher blood supplies. The nucleated cells of endometrium were large and compression was more. The uterus wall was projected into villi thick endometrium and endothelium was very high. In case of combined formulations these all effects were comparatively same, except the effect on corpus luteum of combined formulation was more (Photomicrographs 6.22-6.30).

LIPID PROFILE:

The effects of estrogen replacement therapy on lipid metabolism vary according to the dosage route of administration, type of estrogen and whether concomitant progestogen is given. Generally administration of oral estrogen replacement therapy leads to decreased plasma concentrations of total and low-density lipoproteins (LDL)-cholesterol (Chetkowski *et al.*, 1986). Although such alterations in the lipid profile are consistent with a reduction in the risk of coronary heart disease, plasma concentrations of triglycerides (a risk factor of coronary artery disease) may be elevated. Lipid and lipoprotein changes appear to be less marked with transdermal estradiol than with oral estrogens, presumably reflecting the lack of hepatic first pass effects. The influence on lipid metabolism of concomitant sequential progestogen therapy, which was administered in the majority of studies, is not clear. It has been suggested that progestogen might attenuate the beneficial effects of estrogens on lipids (Lobo, 1990).

(a) Cholesterol: Measurements of serum cholesterol levels are useful in evaluation of the risk of coronary arterial occlusion, atherosclerosis, myocardial infarction, liver function, intestinal absorption, and thyroid function and adrenal disease. Increased levels are found most characteristically in primary hyperlipoproteinaemias, in nephritic syndrome, myxoedema, and obstructive jaundice and in diabetes mellitus. Low values are frequently obtained in anaemias, in hemolytic jaundice, in malabsorption syndrome, severe malnutrition, and acute infections and in terminal state. Very low values occur in beta-lipoproteinaemia and to lesser degree in familial hypo beta-lipoproteinaemias.

Principle: The estimation of cholesterol involves the following enzyme catalyzed reaction:

$$\text{Cholesterol ester} \xrightarrow{\text{Cholesterol esterases}} \text{Cholesterol + Fatty acid}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol Oxidase}} \text{Cholest-4-en-3-one} + H_2O$$

$$2H_2O_2 + 4\ \text{AAP} + \text{Phenol} \xrightarrow{\text{4- Aminoantipyrine}} 4H_2O + \text{Quinoneimine}$$

Absorbance of quinoneimine so formed is directly proportional to cholesterol concentration.

Procedure: The treated groups were used for this study. After fifteen days of application of transdermal patch the blood sample were collected for performing this study and the blood sample about (1 ml) was kept undisturbed for 30-45 minutes for clotting purpose. Then the sample centrifuged for 10 minutes at 2000-3000 rpm, for separation of blood plasma and this plasma was separated by using the pipette and stored in glass vials of 5.0 ml capacity at 4°C. These samples of plasma were analyzed by using cholesterol estimation kit supplied by Transasia Bio-medical ltd, Daman. The cholesterol reagent-1 was mixed with reagent-3 and allowed to attain room temperature. Auqa-4(reagent-3) approximately 20 ml was added to the contents of each supplied vials of reagent–1, swirled to dissolve, but not shaken vigorously. The unheamolysed serum was separated from the cells as soon as possible. The samples were stable for 7 days at 2-8°C. The samples were prepared as shown in the table 6.3.

Table 6.3: Preparation of standard and test samples solution for the estimation of cholesterol

Pipette into tubes marked	Blank	Standard	Test
Working reagent	1000 µl	1000 µl	1000 µl
Distilled water	20 µl	----	---
Standard	----	20 µl	----
Sample	----	----	20 µl

The contents of tubes were mixed and incubated for 10 minutes at 37°C. Read the absorbance of standard and each sample was measured at 510 nm (500-540 nm) and the calculated concentration of cholesterol was recorded in table 6.6.

(b) Triglycerides: Triglycerides are a family of lipids absorbed from the diet and produced endogenously from carbohydrates. Measurement of triglycerides is important in the diagnosis and management of hyperlipidaemias. These diseases can be genetic or secondary to other disorders including nephrosis, diabetes mellitus and endocrine disturbances. Elevation of triglycerides has been identified a risk factor for arteriosclerotic disease. Serum or plasma samples free from hemolysis are suitable for triglyceride estimation. Storage at room temperature may cause the release of glycerol from phospholipids with a resulting apparent increase in triglycerides and hence is not recommended. The samples were prepared in following manner as shown in table 6.4:

Table 6.4: Preparation of standard and test samples solution for the estimation of triglycerides

Pipette into tubes marked	Blank	Standard	Test
Working reagent	1000 µl	1000 µl	1000 µl
Distilled water	10 µl	----	---
Standard	----	10 µl	----
Sample	----	----	10 µl

The contents were mixed and incubate for 10 minutes at 37°C. The absorbance of standard and each sample was measured at 510 nm (500-540 nm) and the calculated concentration of triglycerides was recorded in table 6.6.

(c) HDL-Cholesterol: High-density lipoproteins contain particles of different densities including lipid and highest concentration of proteins amongst the different lipoproteins. It include free and esterified cholesterol, triglycerides, phospholipids and apolipoproteins A, C, and E. HDL-Cholesterol values are about $1/5^{th}$ of the total cholesterol values and can be expressed as percentage of total cholesterol. Samples were mixed with precipitating reagent and allowed the mixture to stand for 10 minutes at room temperature. It was centrifuged at 4000 rpm for 10 minutes to obtain a clear supernatant. The supernatant was used to determine the concentration of HDL cholesterol in the sample, and then the samples were prepared in following manner shown in table 6.5.

Table 6.5: Preparation of standard and test samples solution for the estimation of HDL-cholesterol

Pipette into tubes marked	Blank	Standard	Test
Cholesterol working reagent	1000 µl	1000 µl	1000 µl
Distilled water	50 µl	----	---
HDL-Standard	----	50 µl	----
Sample	----	----	50 µl

The contents were mixed and incubate for 10 minutes at 37°C. The absorbance of standard and each sample was measured at 510 nm (500-540 nm) and the calculated concentration of HDL-cholesterol was recorded in table 6.6.

Table 6.6: Lipid profile of different groups treated with proniosomal gels formulation of single and combined drugs

S. No.	Control	LN	17β	EEE	17β+LN	EEE+LN
CHL	159.12	173.84	150.40	137.32	183.65	132.97
	173.84	189.10	146.04	129.15	168.93	137.32
	164.03	183.65	150.40	132.32	173.84	137.32
	154.76	178.74	146.04	124.79	168.93	132.97
	159.12	173.84	150.40	129.15	164.03	132.97
	150.40	183.65	137.32	141.89	178.74	124.79
Tg	213.51	156.75	187.38	181.08	219.81	187.38
	271.03	187.38	168.46	163.06	213.51	187.08
	234.23	219.81	151.35	200.00	187.88	187.38
	227.02	187.38	181.08	187.38	206.30	193.69
	219.81	200.00	174.77	168.46	200.00	200.00
	271.03	187.38	163.06	174.77	193.69	187.38
HDL-CHL	59.55	75.00	71.32	52.20	75.00	37.50
	67.64	83.82	63.23	48.52	87.49	41.17
	59.55	83.82	63.23	52.20	79.41	48.52
	48.52	79.41	75.00	54.41	83.82	44.85
	63.23	83.82	67.64	59.55	79.41	52.02
	67.64	79.41	59.55	52.20	75.00	41.17
VLDL	42.70	31.35	37.47	36.21	43.96	37.47
	54.20	37.47	33.69	32.61	42.70	37.41
	46.84	43.96	30.30	40.00	37.57	37.47
	45.40	37.47	36.21	37.47	41.26	38.73
	43.96	40.00	34.95	33.69	40.00	40.00
	54.20	37.47	32.61	34.95	38.73	37.47
LDL	56.87	67.49	41.85	48.91	64.79	62.40
	52.00	67.71	49.12	48.02	38.74	55.59
	57.64	55.87	56.90	40.00	56.86	51.33
	60.84	61.76	36.09	32.51	43.85	49.39
	51.93	50.02	46.19	35.91	44.62	40.77
	32.51	66.67	45.16	54.74	65.01	46.15

Mean Value with standard deviation (n=6)

S. No.	Drug	CHL	Tg	CHL-HDL	VLDL	LDL
1	Control	160.21±8.10	239.43±25.43	61.02±7.11	47.88±5.08	51.96±11.60
2	LN	180.47±6.09	189.78±20.56	80.88±3.60	37.95±4.11	61.58±7.27
3	17β	146.76±5.09	171.01±12.95	66.66±5.76	34.20±2.58	45.88±6.99
4	EEE	132.43±6.21	179.12±13.40	53.18±1.49	35.82±1.09	43.34±8.56
5	17β+LN	173.02±7.22	203.53±12.05	80.02±4.92	40.70±2.41	52.31±11.42
6	EEE+LN	133.05±4.57	190.48±5.31	44.20±5.35	38.09±1.06	50.93±7.50

Table 6.7: Mean Value of lipid profile shown in table

S. No.	Drug	CHL	Tg	CHL-HDL	VLDL	LDL
1	Control	160.21±8.10	239.43±25.43	61.02±7.11	47.88±5.08	51.96±11.60
2	LN	180.47±6.09	189.78±20.56	80.88±3.60	37.95±4.11	61.58±7.27
3	17β	146.76±5.09	171.01±12.95	66.66±5.76	34.20±2.58	45.88±6.99
4	EEE	132.43±6.21	179.12±13.40	53.18±1.49	35.82±1.09	43.34±8.56
5	17β+LN	173.02±7.22	203.53±12.05	80.02±4.92	40.70±2.41	52.31±11.42
6	EEE+LN	133.05±4.57	190.48±5.31	44.20±5.35	38.09±1.06	50.93±7.50

Calculation:

$$\text{(A) Cholesterol (mg/dl)} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \times \text{Concentration of standard (mg/dl)}$$

$$\text{(B) Triglycerides (mg/dl)} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \times \text{Concentration of standard (mg/dl)}$$

$$\text{(C) HDL Cholesterol (mg/dl)} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \times \text{Value of standard} \times \text{Dilution Factor (mg/dl)}$$

$$\text{(D) VLDL} = \frac{\text{Plasma (Serum) Triglycerides}}{5}$$

$$\text{(E) LDL} = \text{(Total serum Cholesterol-HDL)} - \text{(VLDL)}$$

RESULT AND DISCUSSION

Estrogen replacement therapy has been proposed to reduce the risk of cardiovascular disease in postmenopausal women, partly through the effects on lipoprotein metabolism. In oral estrogen replacement therapy the plasma concentration of low-density lipoprotein LDL cholesterol decreased, while HDL cholesterol increased (Chetkowski *et al.*, 1986). Such changes are less prominent if the treatment is administered with subcutaneous gestrogen therapy but increases the risk of endometrial malignancy. This risk is, however, reduced if estrogen treatment is combined with gestagens. The most commonly used types of gestagens, 19-norprogestogens are known to reduce the HDL-Cholesterol fraction when given orally (Richard *et al.*, 1992).

In the present study various groups of animals were treated with transdermal proniosomal gel containing, only estradiol, levonorgestrel, ethinylestradiol, and two combined formulations containing estradiol + levonorgestrel, and ethinylestradiol + levonorgestrel. One group was kept as control. Mean value of plasma concentration of plasma lipids and lipoproteins are presented in table 6.7.

S. No.	Drug	CHL	Tg	CHL-HDL	VLDL	LDL
1	Control	160.21±8.10	239.43±25.43	61.02±7.11	47.88±5.08	51.96±11.60
2	LN	180.47±6.09	189.78±20.56	80.88±3.60	37.95±4.11	61.58±7.27
3	17β	146.76±5.09	171.01±12.95	66.66±5.76	34.20±2.58	45.88±6.99
4	EEE	132.43±6.21	179.12±13.40	53.18±1.49	35.82±1.09	43.34±8.56
5	17β+LN	173.02±7.22	203.53±12.05	80.02±4.92	40.70±2.41	52.31±11.42
6	EEE+LN	133.05±4.57	190.48±5.31	44.20±5.35	38.09±1.06	50.93±7.50
n = 6						

As shown in table above, plasma cholesterol level in comparison to control group increased in the group that was treated with proniosomal gel containing Levonorgestrel but decreased with proniosomal gel containing estradiol and estradiol + levonorgestrel. The lower value was found in the case of proniosomal gel containing ethinylestradiol and ethinylestradiol+ levonorgestrel. As reported in the literature also that the progestrogens is necessary to reduce the endometrial adverse effects hence the combination of

ethinylestradiol+ levonorgestrel may be the best therapy for affecting the lipid cholesterol level. The overall effect of drugs used in formulations on the cholesterol level may be expressed in decreasing order (Figure 6.55);

$$T1_{(LN)} > T1_{(17\beta+LN)} > T1_{(Cont)} > T1_{(17\beta)} > T1_{(EEE+LN)} > T1_{(EEE)}.$$

The increased level of triglycerides has been identified a risk factor for atherosclerotic disease and its management is also important in the hyperlipidaemia. In this study the plasma triglycerides level in comparison to control group decreased in all formulations and decreasing level of triglycerides were found in following order (Figure 6.56);

$$T1_{(Cont)} > T1_{(17\beta+LN)} > T1_{(EEE+LN)} > T1_{(LN)} > T1_{(EEE)} > T1_{(17\beta)}.$$

The plasma HDL-Cholesterol level was decreased in the group that was treated with proniosomal gel containing ethinylestradiol with or without levonorgestrel except plain levonorgestrel proniosomal gel had shown increasing effect. The effect of formulations on HDL cholesterol level was found in decreasing order as follows (Figure 6.57);

$$T1_{(LN)} > T1_{(17\beta+LN)} > T1_{(17\beta)} > T1_{(Cont)} > T1_{(EEE)} > T1_{(EEE+LN)}$$

The Lipoprotein subfractions i.e. plasma VLDL decreased with all the formulation of proniosomal gels containing estradiol, ethinylestradiol, levonorgestrel and their combinations. The VLDL level was found in decreasing order (Figure 6.58);

$$T1_{(Cont)} > T1_{(17\beta+LN)} > T1_{(EEE+LN)} > T1_{(LN)} > T1_{(EEE)} > T1_{(17\beta)}$$

The LDL level was increased in all the treated groups in comparison to control group. The influence of different formulations was found in decreasing order as follows (Figure 6.59);

$$T1_{(LN)} > T1_{(17\beta+LN)} > T1_{(Cont)} > T1_{(EEE+LN)} > T1_{(17\beta)} > T1_{(EEE)}$$

All these results clearly indicates that the levonorgestrel alone has increasing effects on lipid profile on the treated animals and its combination with estradiol also has the similar effects but to a lesser extent. However the combination of levonorgestrel with ethinylestradiol had shown good results on the lipid profile of treated animal. Therefore proved to be a better combination for both contraception and hormone replacement therapy given in the proniosomal gel formulation through the transdermal route.

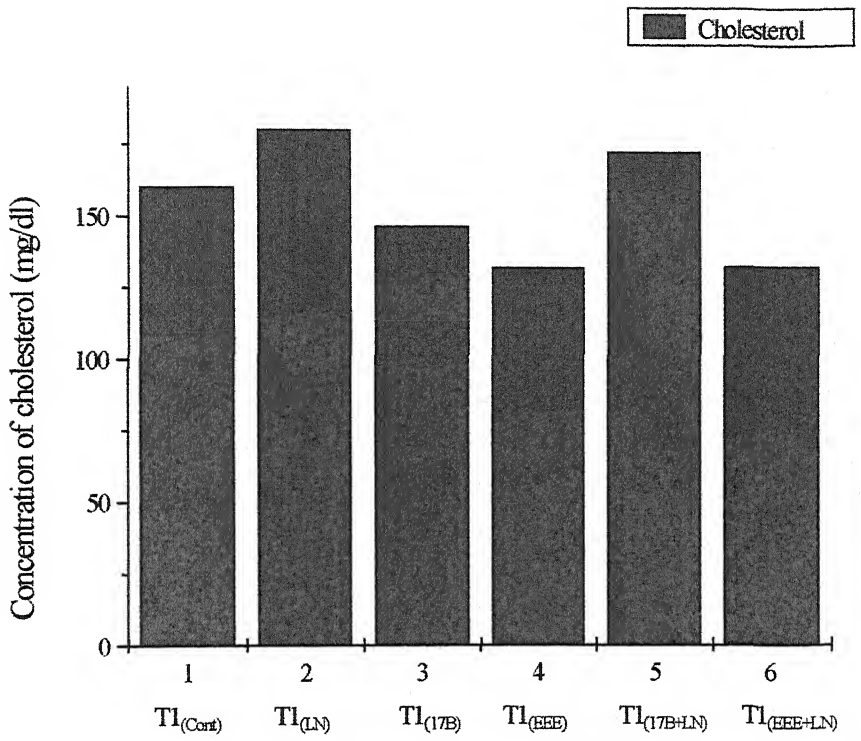


Figure 6.55: Blood cholesterol level in treated animals with single drug formulation T1 $_{(LN)}$, T1 $_{(17\beta)}$, T1 $_{(EEE)}$, and combined drug formulation T1 $_{(17\beta+LN)}$, & T1 $_{(EEE+LN)}$ formulations.

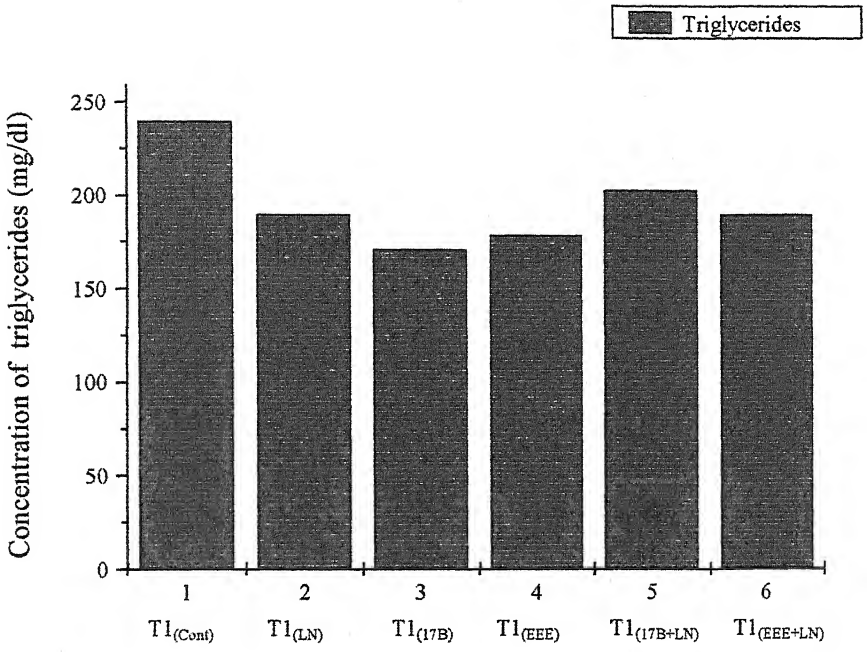


Figure 6.56: Blood triglycerides level in treated animals with single drug formulation T1 $_{(LN)}$, T1 $_{(17\beta)}$, T1 $_{(EEE)}$, and combined drug formulation T1 $_{(17\beta+LN)}$, & T1 $_{(EEE+LN)}$ formulations.

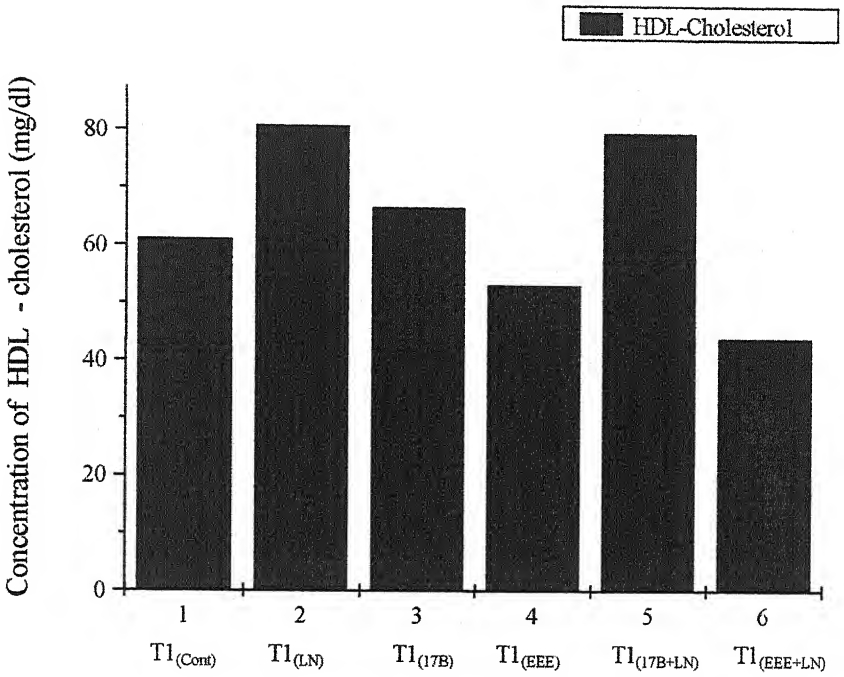


Figure 6.57: Blood HDL-cholesterol level in treated animals with single drug formulation T1 $_{(LN)}$, T1 $_{(17\beta)}$, T1 $_{(EEE)}$, and combined drug formulation T1 $_{(17\beta+LN)}$, & T1 $_{(EEE+LN)}$ formulations.

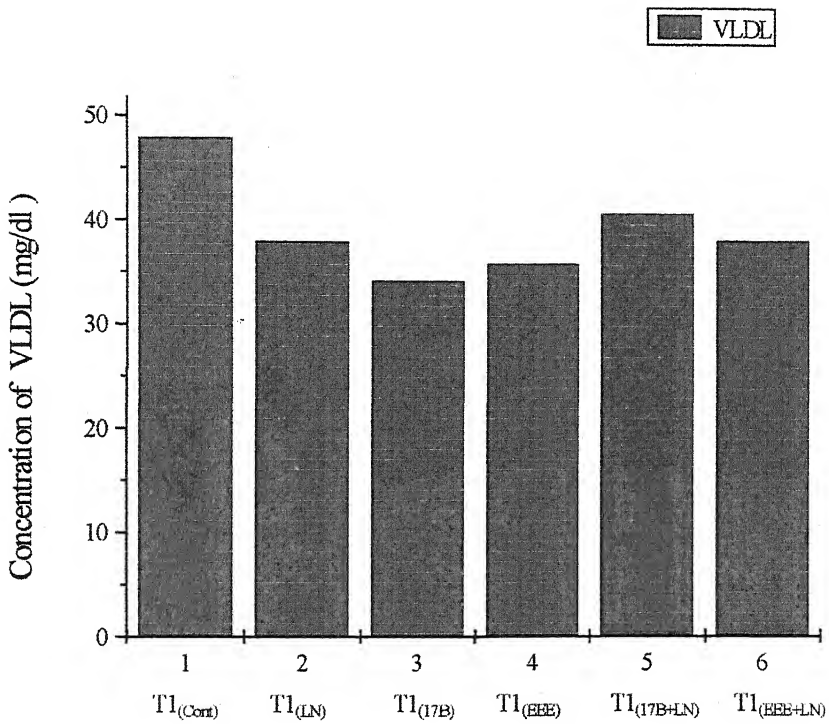


Figure 6.58: Blood VLDL level in treated animals with single drug formulation T1 $_{(LN)}$, T1 $_{(17\beta)}$, T1 $_{(EEE)}$, and combined drug formulation T1 $_{(17\beta+LN)}$, & T1 $_{(EEE+LN)}$ formulations.

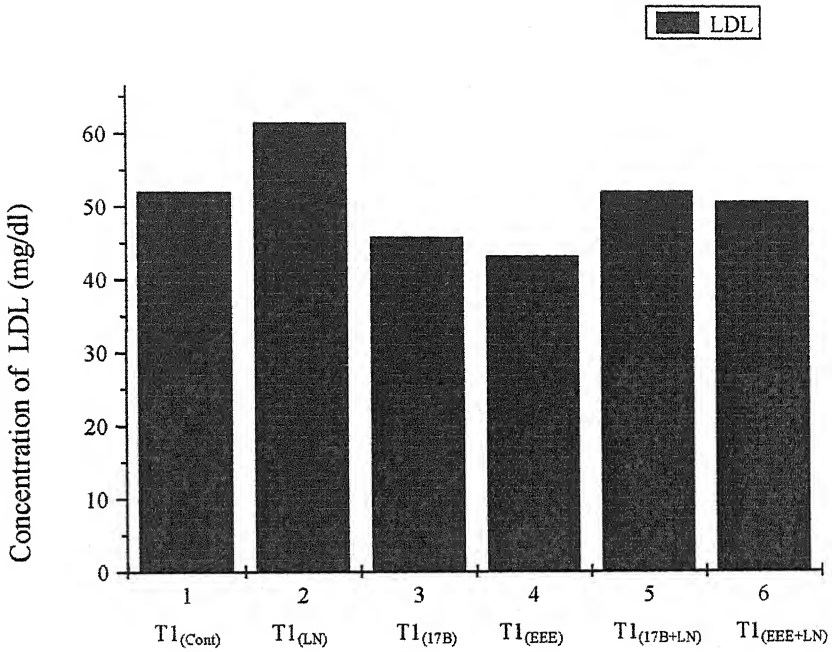


Figure 6.59: Blood LDL level in treated animals with single drug formulation T1 $_{(LN)}$, T1 $_{(17\beta)}$, T1 $_{(EEE)}$, and combined drug formulation T1 $_{(17\beta+LN)}$, & T1 $_{(EEE+LN)}$ formulations.

Chapter-7

SUMMARY AND CONCLUSION

With increasing life expectancy, many women will spend more than one-third of their life span in the postmenopausal state. Increasing awareness of the problems of postmenopausal osteoporosis and cardiovascular disease, together with recognition of the lessened risk of endometrial hyperplasia when sequential progestogen is coadministered with estrogen, has led to a reevaluation of the risk-benefit ratio of estrogen replacement therapy.

It has been suggested that the transdermal estradiol in combination with a sequential progestogen might also offer a better-tolerated means of contraception than oral estrogens.

Growing evidence demonstrates that the transdermal route offers several potential advantages over conventional routes for systemic medication e.g. avoidance of first pass elimination, extended duration of therapeutic activity with reduced side effects and patient compliance. Research has been persuaded to enhance the permeability of drug across the stratum corneum and to achieve higher systemic concentration of drugs.

Several types of transdermal therapeutic systems, which utilize the rate controlled drug delivery technologies to modulate the transdermal systemic delivery of therapeutic agent have been successfully developed and commercialized.

Although a few vesicular systems like liposomes and niosomes have been studied for contraception through transdermal routes, their unstable nature limits their use in TDDS. Therefore, in order to increase the stability of liposomes, concept of proliposomes has been proposed. In a similar approach some of the researchers studied niosomes, which exhibit superior stability and are free from other limitations of liposomes like fusion, drug leakage etc.

The literature survey revealed that no investigation had been reported to date on the use of proniosomes to achieve a combined delivery of estradiol plus levonorgestrel and ethinylestradiol plus levonorgestrel from the same unit of proniosomal transdermal therapeutic system.

This project was designed to investigate the possibility of manufacturing proniosomes as drug carriers for transdermal delivery of drugs. Therefore it was thought worthwhile to incorporate proniosomes in TDDS for contraception and hormone replacement therapy. This is possible because optical anisotropic proniosomes seems to convert into the niosomes *in situ* by absorbing water from the skin.

Estimation of drugs was done in-vitro by UV spectrophotometric method. The analysis of estradiol, ethinylestradiol, and levonorgestrel was performed individually and in combined formulations by multicomponent mode. The scanning of these drugs in 40% v/v PEG 200 solution showed the absorption maxima at 280, 281, and 247 nm respectively. The standard curves of the drugs for spectrophotometric estimation were prepared in a range of 1-10 µg/ml in 40% v/v PEG 200, For combined formulations of these drugs, two combination of standard solution were formed and multicomponent mode was followed for the analysis of drugs. The method was found suitable for determination of drug content in combined formulations containing 2-20 µg/ml concentration of each drug in 40% v/v PEG 200 solution. The amount found experimentally by the multicomponent mode was in the same range as was taken.

The interference of formulation additives in the estimation of drugs was also checked. The absorbance of drug solution was recorded in the presence of maximum concentration of additives used in formulations. No or neglible influence on the absorbance of drug solutions was observed at 280, 281 and 247nm for estradiol, ethinylestradiol and levonorgestrel respectively.

The drug samples were identified by the I. R. spectroscopy. The I. R. spectrum of estradiol, ethinylestradiol, and levonorgestrel were found to be similar to those reported in B P 1998 and confirmed the identitity of these drugs.

In preformulation studies, the solubility determination of estradiol, ethinylestradiol and levonorgestrel was performed in common aqueous and organic solvents. Estradiol, ethinylestradiol, and levonorgestrel showed good solubility in alcohol, ether, PEG-200, acetonitrile and they were found to be sparingly soluble in chloroform and insoluble in water.

The partition coefficients of estradiol, ethinylestradiol, and levonorgestrel in n-octanol : water system were found to be 2.062, 1.074, and 0.893 respectively, which are indicative of lipophilic nature of drugs. The lipophilic nature was found in following order estradiol > ethinylestradiol >levonorgestrel.

The preliminary formulations of proniosomal gels were designed using combinations of spans only. These formulations showed maximum release rate upto 60-65%. Therefore in order to optimize the drug release, the proniosomal gel formulations were designed by using the combinations of span with tweens in addition to

span-span combination for achieving the desired transdermal flux of estradiol, ethinylestradiol and levonorgestrel.

Initially the proniosomal gel were developed with combinations of tweens with or without cholesterol as per method reported by Perrett *et al.* (1990). But these combinations fail to produce proniosomal gel. Freely soluble non-ionic surfactant such as tween can form the micelles on hydration and in the presence of cholesterol above 30% concentration of the selected ratio of tweens, it produced only liquid crystals either square shape or needle shape. This is because the vesicles cannot be formed by tweens only.

Proniosomal gels were prepared by drop wise addition of water to lipid dissolved in alcohol by heating. The addition of water resulted in the precipitation of lipid in the form of hydrated bilayers. Similarly proniosomal gels were also prepared by addition of water to warmed solution of surfactants (Span + Tween) in alcohol. An initial proniosomal gel was prepared consisting of lipid or surfactant: alcohol: water in the proportion of surfactant (300mg Span 40: 100 mg Tween20): alcohol (0.75 ml): water (9 drops). This proniosomal gel was used as a control formulation.

All these formulations were evaluated for their visual and microscopic appearance under cross polarizer microscope for optical anisotropic structure, and from amongst them optimized formulations were utilized for the characterization like consistency, crystal appearance, shape and size, encapsulation efficiency, release rate through cellophane membrane and rat skin. Niosomes derived from proniosomes were also characterized for their size and size distribution after hydration with or without agitation and with sonication. The niosomes were also evaluated for polydispersity index, and drug entrapment efficiency.

These formulations were developed with span 20 (3) with tween 20, 40, 60, 80 (1), span 40 (3) with tween 20, 40, 60, 80 (1), span 60 (3) with tween 20, 40, 60, 80 (1), span 80 (3) with tween 20, 40, 60, 80 (1), span 40 (3) with tween 20 (1) containing different alcohols, span 40 (3), tween 20 (1), and soya lecithin, egg lecithin, dicetylphosphate, cholesterol (1). All of these formulations were containing ethinylestradiol. The same formulations were developed with estradiol and levonorgestrel.

These proniosomal gels were chosen on the basis of (a) optical anisotropic structure observed under the cross polarizer equipped microscope and (b) formation of vesicles spontaneously upon hydration. More than 100 proniosomal formulations using

combinations of surfactants (Spans & Tweens) and alcohols were prepared in the same way. Out of these, three formulations prepared using the combination of Span 40 and tween 20, Span 40, tween 20 and lecithin, and Span 40, tween 20 with cholesterol were selected for further studies because they showed the uniform vesicle size with good entrapment efficiency.

Niosomes derived from proniosomes indicated that the process of dissolution may occur by progressive hydration of surfactant on the surface of proniosomes taking the form of niosomes as "budding off" from the outer surface of proniosomal gel. So long as the hydration was taking place, the formation of vesicles was continued. This may be due to the presence of tween with span that could produce the hydration environment for more water absorption. It seems likely that the comparatively uniform size of niosomes formed under this relative static condition may result in the absence of shear force normally present during conventional hydration procedure. The size of vesicles formed was larger in case of hydration without agitation while it was smaller in case of hydration with agitation. This may be attributed to breaking of vesicles due to shear force of agitation. The diameter of most niosomes appears to lay in the range of 1.52 μm to 15.44 μm; little variation of size was seen between the batches of niosomes of given composition. The diameter of niosomes in formulation of span40: span20 was in the range of 8.84 μm to 15.44 μm, and in formulations ST22, ST24, ST26, ST28 was 3.01 μm to 3.82, in ST42, ST44, ST46, ST48 was 5.44μm to 6.63, and in formulations ST62, ST64, ST66, ST68 it was 6.02μm to 6.84 μm, and in ST82, ST84, ST86, ST88 was 4.12 μm to 5.22 μm. The size of niosomes prepared using different lipids was in range of 4.33 μm to 7.99 μm. However the size of vesicles formed from proniosomal gel with cholesterol was uniform and very small. The polydispersity index was always found to be very low showing that this method of niosome formation results in vesicles of highly uniform in size. In the case of span-span combinations (HLB value 7.08 to 7.65) the vesicle size was larger (8.44 μm -15.44 μm) in comparison to formulation prepared using span tween combination (HLB value 8.75 to9.15). But span 40 + tween 20 produced medium size (5.44 μm to 6.63 μm) uniform vesicles. Therefore formulations containing span 40 with tweens were considered to be optimum because incorporation of tween had reduced the size of vesicles.

The phase transition temperature of span 40 is higher in comparison to span 20, which affects the permeability of bilayers. Therefore, as the proportion of span 40 was changed, the permeability also changed. Thus the size was found to decrease with increasing HLB value of surfactant mixture but drug entrapment efficiency also decreased in the same order.

In the case of different combinations of span and tween the size of vesicle was decreased with increasing HLB value. But the drug entrapment efficiency was more in case of span- span combination while it was less in case of combination of span with tween.

These results indicated that the drug entrapment (estradiol, ethinylestradiol and levonorgestrel) was more in the case of surfactants combination of low HLB value (hydrophobic) and decreased as the HLB value of surfactant combination was increased. This may be accounted for the hydrophobic nature of drug having more affinity towards a hydrophobic surfactant span.

The entrapment efficiency was higher in case of spans combination and overall entrapment efficiency was in decreasing order of SS42 > ST42 with lipid > ST22 > ST42 >ST 62 > ST82. (where S= span, T=tweens, 2 = 20, 4= 40, 6= 60, 8=80).

The microscopic study of niosomes revealed that the vesicle size was in decreasing order of

SS42 > ST42 with lipid > ST62 > ST82 >ST 42 > ST22. This again may be attributed to hydrophilic nature of tweens forming small vesicles on hydration with water.

From different compositions of lipids, the vesicle size in niosomes was found to be in the increasing order of ST42CHL < ST42DCP < ST42 EL < ST42 SL. This may be attributed to the varying factors like change in solubility, effect of bilayer integrity and their intrinsic composition. In the case of egg and soya lecithin, a proper conclusion could not be made. The entrapment efficiency of niosomes prepared using lipids was found in the increasing order of ST42EL < ST42SL < ST42 DCP < ST42 CHL. This order reflected the rigidization effects of various additive lipids. The lipid causing the highest rigidization of bilayer i.e. cholesterol shows highest entrapment efficiency .The study of vesicle size and percent (%) drug entrapment of different proniosomal formulations revealed that in case of large vesicle percent (%) of drug entrapment was more as compared to small vesicles.

Vesicles formed from different alcohols were of different sizes. They followed the increasing order in size as Isopropyl alcohol < Absolute alcohol< Propanol < Butanol. Vesicles with isopropyl alcohol results in smallest size which may be due to branched chain present in it and larger size was found with other homologues alcohols.

From the different proniosomal formulations of ethinylestradiol, release rate across cellophane membrane was determined after optimization of formulation. Their permeation rate was also determined through rat skin using locally fabricated Keshary-chein type diffusion cell. The drug release rate was found to follow zero order. On the basis of these release rate observations, formulation ST42 (T1) was selected for further studies in order to see the effect of different amount of drugs, spans, phospholipids, alcohols and sonication time, on drug permeation profile studied. Similarly the release rate of estradiol and levonorgestrel for permeation was also performed in the same manner. After optimization of proniosomal formulation containing estradiol, ethinylestradiol, and levonorgestrel and permeation through cellophane membrane, two combined formulations S1 and T1 were developed and their permeation profile through rat skin was determined by using locally fabricated Keshary-chein type diffusion cell.

All these formulations showed linear correlation between spans and tweens in the proniosomal gel systems for transdermal flux. The transdermal flux pf proniosomal formulation was in following order for mixture of spans and tweens, i.e. ST22>ST82>ST42>ST62. The reason for the increased drug release may be the increase in HLB value with the addition of tween 20, 40, 60, & 80. From all these formulations of span 20 with tween 20 and 80 were found to be in the liquid state while composition of span 40 & 60 with span 20 were in ordered gel state which delayed the drug release.

In order to see the effect of alcohols, the formulation ST42 was prepared with different alcohols and applied to treated cellophane membrane. The apparent steady state transdermal flux was highest for the formulation containing isopropyl alcohol. Inclusion of absolute alcohol, and propanol, and butanol into the formulation also led to the enhanced drug release but the effect was less than isopropyl alcohol. This may be due to the branched chain structure of isopropyl alcohol, which acts as a cosurfactant and might have reduced the bilayer packing with resultant increase in the flux value. The presence of tweens that can easily equilibrate with the alcohols for hydration also enhanced the transdermal flux value. The effect of alcohols on the release rate of drugs from plain

formulation (ST42) prepared with different alcohols was observed in the following decreasing order.

ST42 (Isopropyl alcohol) > ST42 (Absolute alcohol)>ST42 (Propanol) >ST42 (Butanol).

Effect of sonication time on the selected formulation (ST42) the flux was maximum after 120 seconds of ultrasonication. The flux was found to increase with the sonication time. This may be due to the membrane disruption reducing vesicle size and energy mediated mixing of vesicle compositions and skin lipids.

In case of proniosomes prepared using mixture of spans the release rate was found to be in increasing order if the amount of span20 was increased SS24 (ratio 1:1)<SS24 (ratio 2:1)<SS24 (ratio3:1)<SS24(ratio 4:1) and on increasing the ratio of span 40 the release rate was found in the order of SS42(ratio 1:1)>SS42(ratio 2:1)>SS42(ratio 3:1)>SS42(ratio 4:1). Among all the combinations of spans; span 40 & tweens the release rate was found to be in the following decreasing order in case of T20, 40, 60, 80 with span 20 > T20, 40, 60, 80 with span 80 > T20, 40, 60, 80 with span 40 > T20, 40, 60, 80 with span 60. This was in accordance with the phase transition temperature of the surfactant mixtures. The effect of different spans on the release rate showed that the maximum flux was in the case of span 20 and span 80 because span 20 and 80 has low phase transition temperature. The minimum flux was obtained in the case of span 40 and span 60. Further the slow release may be due to the reduction in surface free energy that caused the formation of larger size vesicles resulting in smaller surface area exposed to receptor medium and membrane or skin. The low phase transition temperature of span 20 combinations allowed the faster release than span 80 combinations.

All the formulations containing span and tween combinations released significantly less estradiol, ethinylestradiol than phospholipid formulation. The release rate was in the following decreasing order of :

ST42SL >ST 42DCP > ST42 EL > STCHL>ST24.

In the case of formulation with varying composition of lipids the drug release was maximum in soya lecithin. The dicetylphosphate formulation showed intermediate release because its vesicle are charged which are responsible for increasing the curvature and decreasing the size of vesicle with the increase in surface area. Both the commercial soya lecithin and egg lecithin were effective penetration enhancers. The drug release rate was more in soya lecithin formulation than egg lecithin. Higher skin permeability may be due to an increase in partition coefficient between vehicle and skin or direct effect of

lecithin on the skin thereby reducing the skin resistance to permeation of the drugs. Both soya and egg lecithin contain phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol and polyunstarated acids including lenoleic acid and lenolenic acid. These polyunsaturated fatty acids are relatively more in soya lecithin that may be the reason for faster release rate than egg lecithin. The cholesterol increases the rigidity of bilayers hence slowest release rate was observed in formulations containing cholesterol.

On the basis of these release rate studies, formulation ST42 was found to exhibit optimum drug release and permeation through the membrane. The formulation containing estradiol, ethinylestradiol, and levonorgestrel were prepared separately with or without tweens and lipids and evaluated for drug release studies. The effect of soya lecithin, egg lecithin, dicetylphosphate and cholesterol on the drug release rate from the formulations was also studied.

After optimization of these formulations containing single drug, two formulations of span: span combination (span 40 and span 20) were prepared containing drug combination i.e. estradiol with levonorgestrel (1mg+1mg) and ethinylestradiol with levonorgestrel (1mg+1mg) and coded as S-1 and S-2. The formulations of span 40 with tween 20 were prepared containing same drug combinations and coded as T-1 and T-2. The release rate was found higher in the case of T-1 and T-2. This may be due to the higher transdermal flux of span and tweens formulation in comparison to spans combination only. This may be due to the better hydration of proniosomal gel with resultant smaller vesicles in presence of tween.

Stability study was done on proniosomal gel formulations SS 42 (S1) and ST 42 (T1) and ST42 with cholesterol (T_{CH}). These formulations were stored at 4°C, 25±2°C and 40±2°C in clean glass vials. Prior to hydration, the appearance of crystals and size were determined by observing under microscope. Drug crystals were not seen after 12 weeks in case of formulation SS 42 (S1) and ST 42 (T1) while in case of ST42 with cholesterol (T_{CH}), crystals were not observed even after 16 weeks. The consistency of proniosomal gel SS42 (S1) was increased after 12 weeks storage and in case of ST42 CHL ($T1_{CH}$) consistency was increased after 16 weeks. There was no significant difference in the optical anisotropic structure of proniosomal gel formulation even after 16 weeks of storage time.

The polydispersity analysis of formulations SS42 (S1), ST42 (T1), and $ST42_{CHL}$ (T_{CH}) was performed and niosomes were counted. The results of polydispersity index showed that the vesicle size increased and polydispersity index decreased.

In vivo performance of optimized proniosomal transdermal gel system was studied. The estrogenic activity was determined in terms of their effect on uterine weight, vaginal opening and cornification. Formulation $T1_{(17\beta)}$ and $T1_{(EEE)}$ was applied on the skin of dorsal side of the animal. In the smear of immature rats, the epithelial cells were comparatively bigger in the case of $T1_{(EEE)}$ than $T1_{(17\beta)}$ in comparison to control animals. After four-day application of patch, only keratinized cells without any nucleus were seen. This was clear indication that ethinylestradiol has produced full cornification in comparison to estradiol. The other formulations containing levonorgestrel, $T1_{(LN)}$ and combined formulation of ethinylestradiol with levonorgestrel, $T1_{(EEE + LN)}$ & estradiol with levonorgestrel $T1_{(17\beta + LN)}$ showed mixture of leukocytes and epithelial cells. The histological studies showed that the endometrial mucosal thickness in control animals and those treated with proniosomal gel $T1_{(Cont)}$, $T1_{(17\beta)}$, $T1_{(EEE)}$, $T1_{(LN)}$ and combined formulation $T1_{(EEE + LN)}$ & $T1_{(17\beta + LN)}$ were 16.61 μm, 52.62 μm, 58.42 μm, 64.52 μm, 70.12 μm and 76.32 μm respectively. This may be due to the stimulation of protein synthesis by these hormonal drug formulation.

The contraception studies were performed for their endometrium thickness, ovulation point and formation of corpora lutea. The percent inhibition of corpora lutea with proniosomal gel $T1_{(17\beta)}$, $T1_{(EEE)}$ & $T1_{(LN)}$ and combined formulation $T1_{(EEE+LN)}$ & $T1_{(17\beta + LN)}$ was 41.75%, 49.23%, 52.14, 89.41% and 94.74%, and number of ovulation point were less in both combined formulations.

In the plasma lipid studies of various groups of animals treated with transdermal proniosomal gels containing, only estradiol, levonorgestrel, ethinylestradiol, and two combined formulations containing estradiol + levonorgestrel, and ethinylestradiol + levonorgestrel produced favorable changes in lipid components of plasma in rats.

The plasma cholesterol level increased in the animals group which was treated with proniosomal gel containing levonorgestrel but decreased with proniosomal gel containing estradiol and estradiol + levonorgestrel. The lower value was found in the case of proniosomal gel containing ethinylestradiol and ethinylestradiol+ levonorgestrel. The

overall effect of drugs used in formulations on the cholesterol level was observed in decreasing order;

$$T1_{(LN)} > T1_{(17\beta+LN)} > T1_{(Cont)} > T1_{(17\beta)} > T1_{(EEE+LN)} > T1_{(EEE)}.$$

The increased level of triglycerides has been identified a risk factor for atherosclerotic disease and its management is also important in the hyperlipidaemia. In this study the plasma triglycerides level in comparison to control group decreased in all formulations in following order;

$$T1_{(Cont)} > T1_{(17\beta+LN)} > T1_{(EEE+LN)} > T1_{(LN)} > T1_{(EEE)} > T1_{(17\beta)}.$$

The plasma HDL-cholesterol level was decreased in the animal group that was treated with proniosomal gel containing ethinylestradiol with or without levonorgestrel. The plain levonorgestrel proniosomal gel had shown increasing effect on HDL-cholesterol level. The effect of proniosomal formulations on HDL cholesterol level was found in decreasing order as follows;

$$T1_{(LN)} > T1_{(17\beta+LN)} > T1_{(17\beta)} > T1_{(Cont)} > T1_{(EEE)} > T1_{(EEE+LN)}$$

The lipoprotein subfractions i.e. plasma VLDL decreased with all the formulations of proniosomal gels containing estradiol, ethinylestradiol, levonorgestrel and their combinations. The VLDL level was found in decreasing order;

$$T1_{(Cont)} > T1_{(17\beta+LN)} > T1_{(EEE+LN)} > T1_{(LN)} > T1_{(EEE)} > T1_{(17\beta)}$$

The LDL level was increased in all the treated groups of animals except estradiol /ethinylestradiol formulations in comparison to control group. The influence of different formulations was found in decreasing order as follows;

$$T1_{(LN)} > T1_{(17\beta+LN)} > T1_{(Cont)} > T1_{(EEE+LN)} > T1_{(17\beta)} > T1_{(EEE)}$$

The effect of oral administration of hormonal drugs leads to various problem like endometrial cancer, breast cancer, thromboembolic disorder, hypertension. The proniosomal transdermal drug delivery thus offers a convenient and well-tolerated form of estrogen replacement therapy in post menopauasal women seems to provide a week protective effect against cardiovascular disease; this effect had been associated with a decrease of serum low-density lipoprotein cholesterol.

In, conclusion, the result reported here indicates that proniosomes are very promising means as drug carriers. The essential features of proniosomes are their ability to rearrange on dilution to form a stable niosome suspension, which allow faster permeation through the lipid/surfactant bilayers formed by this technique. Drugs from

proniosomes seem to pass through the skin with comparatively faster rate than free drug. The experimental results and supportive theoretical analysis suggest that either direct transfer of drug(s) from vesicles to the skin or the penetration enhancer effect of non-ionic surfactant may contribute to the faster permeation of drugs from proniosomal formulations. Thus proniosomal gel appears to efficiently deliver drugs by the transdermal route as observed in this study. A high proportional of span 40 with tweens (20, 40, 60, 80) was needed in the formulations to enhance the transdermal flux of drugs. While no significant difference was observed on comparing the transdermal flux of different formulations of span 40 with tweens. The proniosomal gels developed with cholesterol, soya lecithin, egg lecithin, and dicetylphosphate showed higher drug entrapment and release rate except with cholesterol. These findings suggest that inclusion of surfactants and lecithin in niosome vesicle derived from proniosomes may play a significant role in drug permeation than inclusion of cholesterol alone. Proniosomes may become a useful dosage form for estradiol, ethinylestradiol and levonorgestrel, specifically due to their simple, scaling-up production procedure and ability to modulate drug transfer across skin.

In this study the proniosomal transdermal replacement therapy reduced total cholesterol and triglycerides, and VLDL while HDL-cholesterol level was increased with proniosomal gel containing levonorgestrel and estradiol with or without levonorgestrel. The level of LDL was also reduced with proniosomal gel containing estradiol, and ethinylestradiol but increased with levonorgestrel. A neglible change was observed with proniosomal formulation containing estradiol/ethinylestradiol with levonorgestrel. All these results clearly indicate that the levonorgestrel alone has increasing effects on lipid profile of the treated animals and its combination with estradiol also has the similar effect but to a lesser extent. However the combination of levonorgestrel with ethinylestradiol had shown good results on the lipid profile of treated animals. Therefore, it proved to be a better combination for both contraception as well as for hormone replacement therapy when given as proniosomal gel formulation through the transdermal route. In human females, the cardiovascular problems will not occur on using proniosomal gels of estradiol, ethinylestradiol with levonorgestrel for contraception as well as for hormone replacement therapy, which are encountered on oral treatment.

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LIST OF CHEMICALS

Estradiol	Sun Pharmaceutical Pvt. Ltd., Vadodara
Ethinylestradiol	Famy Care Ltd., Vadodara
Levonorgestrel	Famy Care Ltd., Vadodara
Span-20	Robert Johnson, Mumbai
Span-40	Robert Johnson, Mumbai
Span-60	Robert Johnson, Mumbai
Span-80	Robert Johnson, Mumbai
Tween-20	SD-Fine Chemicals Ltd., Mumbai
Tween-40	SD-Fine Chemicals Ltd., Mumbai
Tween-60	SD-Fine Chemicals Ltd., Mumbai
Tween-80	SD-Fine Chemicals Ltd., Mumbai
Isopropyl Alcohol	Qualigen Chemicals, Mumbai
Absolute Alcohol	Qualigen Chemicals, Mumbai
Propanol	Qualigen Chemicals, Mumbai
Butanol	Qualigen Chemicals, Mumbai
Cholesterol	Central Drug House Pvt. Ltd., Mumbai
Soya Lecithin	Sigma Chemicals, Co. USA.
Egg Lecithin	Sigma Chemicals, Co. USA.
Dicetyl Phosphate	Sigma Chemicals, Co. USA.
Triton-X-100	SD-Fine Chemicals Ltd., Mumbai
PEG-200	Loba Chemical, Mumbai
Acetonitrile	E. Merck India Ltd., Mumbai
n-Octanol	Sisco Chem Industries, Mumbai
Chloroform	Ranbaxy India Pvt. Ltd., Mumbai
Ether	Ranbaxy India Pvt. Ltd., Mumbai
Methanol	Ranbaxy India Pvt. Ltd., Mumbai
Acetone	Ranbaxy India Pvt. Ltd., Mumbai
Potassium Hydrogen Phosphate	E. Merck India Ltd., Mumbai
Sodium Dihydrogen Phosphate	E. Merck India Ltd., Mumbai
Sulphuric Acid	Ranbaxy India Pvt. Ltd., Mumbai
Sodium Chloride	E. Merck India Ltd., Mumbai
Quinol	Qualigen (Galaxo India Ltd. Div.) Mumbai
Lipid Estimation Kit	Transasia Bio-medical Ltd, Daman

ABBREVIATION

2	Span / Tween 20
4	Span / Tween 40
6	Span / Tween 60
8	Span / Tween 80
S	Span
T	Tween
TT	Tween and Tween Combination
ST	Span and Tween Combination
CHL	Cholesterol
DCP	Dicetyl Phosphate
EL	Egg Lecithin
SL	Soya Lecithin
IPA	Isopropyl Alcohol
AA	Absolute Alcohol
P	Propanol
B	Butanol
S1	Span40 + Span 20 (3:1)
T1	Span40 + Tween 20 (3:1)
$T1_{(CHL)}$	Span40 + Tween 20 + Cholesterol (3:1:1)
Cont.	Control
EEE	Ethinylestradiol
17 β	17 β-Estradiol
LN	Levonorgestrel
EEE + LN	Ethinylestradiol plus Levonorgestrel
17 β + LN	Estradiol plus Levonorgestrel
CHL	Cholesterol
Tg	Triglycerides
HDL-CHL	HDL-Cholesterol
VLDL	Very low-density lipoprotein
LDL	Low density lipoprotein